Characterisation of the Expression Patterns and Regulation of Juvenile Hormone Esterase and Juvenile Hormone Epoxide Hydrolase Activities in *Drosophila melanogaster* (Diptera)

by

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A thesis submitted for the degree of Master of Science
of The Australian National University, December 1998

Declaration

The research carried out in the course of this investigation and the results presented in this thesis are, except where acknowledged, the original work of the author.

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Acknowledgments

I would like to thank my supervisors, Dr Marion Healy and Dr John Oakeshott, of the Division of Entomology, CSIRO and Dr Paul Cooper, of the Australian National University, for their tireless support, encouragement and interest during the course of my studies and for teaching me so much about insect physiology and statistics. I would like ackowledge that this work was partly funded by an APA Scholarship.

I would also like to thank Dr Peter Campbell, Dr Tara Sutherland and Dr Lynn Riddiford for their helpful discussions and interest in my studies. I am also grateful to the people who helped me proof read this thesis; my supervisors and also Tara, George and Peter.

I would especially like to thank my husband George for his encouragement and support, in times of crisis and in the good times as well, during the writing of this thesis.

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Abstract

The roles and regulation of JH hydrolytic enzymes in *Drosophila melanogaster* were investigated firstly by determining the expression of JHE and JHEH activities from ecdysis to the last larval instar to three hours after pupation and in male and female adults at eclosion to two days old. The developmental profile of JH hydrolysis was similar to results recorded in previous studies in *D. melanogaster* and also similar to other insects. There were two peaks of JH hydrolysis in larval and pupal life stages; the prewandering peak was composed mainly of JHEH activity and the prepupal peak of JHE activity. In adults, JHE specific activity was high at eclosion but declined to relatively low levels by one day old. JHEH specific activity in adults, was uniformally low over the days examined. The spatial expression of JHE and JHEH was investigated in one day old female and male whole adults, segments and tissues. JHE was the more abundant enzyme in both sexes and had its greatest activity in the abdomen, in particular in the reproductive tissues and digestive systems. JHEH activity was highest in the digestive systems of both sexes.

JHE and JHEH activities were regulated developmentally, spatially, sex-specifically and differentially. The JH-JHE feedback mechanism present in lepidopteran larvae was investigated in larval/pupal and adult life stages of *D. melanogaster*. In contrast to lepidopteran species, this feedback mechanism was observed in adult life stages rather than larval/pupal life stages. JH and methoprene treatment of newly eclosed virgins increased JHE activity. Increases ranged from 1.2- to 2.3-fold in whole males and 1.1- to 2.4-fold in whole females. The segments and tissues responsible for these increases in activity were not those in which JHE activity appeared highest in untreated adults. Instead, the segments and tissues with the greatest increases in JHE activity after treatment were those measured to have relatively low levels of JHE activity in untreated adults, including head tissues and haemolymph. This result suggests that either there is some limit to the amount of JHE activity that can be produced tissue specifically or that JHE activity is regulated differentially in tissues, depending on the concentration of free JH present. JH regulation of JHE activity

appeared to be developmentally, spatially and sex specific. JHEH activity was not affected by JH or methoprene treatment in any life stage tested suggesting that JHEH activity is not regulated by JH.

Chapter One

General Introduction

Juvenile hormones are sesquiterpenoid compounds found in arthropods and have been implicated in a diverse range of processes, including behaviour, development and reproduction (Goodman, 1990; Riddiford, 1994). The role in which JH has been most studied is that of metamorphosis, where it interacts with the steroid hormone, ecdysone, to determine the nature of the larval and pupal moults. The classic role ascribed to JH has been to maintain the larval characteristics of insects until transition to the pupal stage when JH titre is decreased to very low levels, allowing metamorphosis to occur (Riddiford, 1994).

Most models of endocrine regulation presume that the titre of circulating hormone is controlled by biosynthesis while the degradation of the hormone remains constant (Hammock, 1985). However, in insects, the circulating titre of JH is believed to be regulated by a number of factors including biosynthesis, degradation, and the titres of binding proteins and receptors (Hammock, 1985; Jones, 1995). Degradation of JH can occur by juvenile hormone esterase (JHE) hydrolysing the ester group to produce JH acid and by juvenile hormone epoxide hydrolase (JHEH) hydrolysing the epoxide group to produce JH diol. A conjugation reaction also occurs in some insects, such as lepidopterans, where JH acid is hydrolysed to JH acid-diol by JHEH, although this reaction is not believed to occur in dipterans (Hammock, 1985; Roe and Venkatesh, 1990; Campbell *et al.*, 1992).

The major enzymes involved in the hydrolysis of JH in *Drosophila melanogaster* are JHE and JHEH (Campbell *et al.*, 1992). The regulation of these enzymes and their roles in regulating JH titre are not well understood. This study focuses on the roles and regulation of JHE and JHEH activities in *D. melanogaster*. It particularly focuses on whether JH regulates one or both of these enzymes at defined life stages and in defined tissues and the implications

that regulation by JH may have for the stage and tissue specific roles of JHE and JHEH activities. The results from this study in a dipteran are compared with information on the role of the JH hydrolysing enzymes in lepidopterans and regulation of their activities by JH.

The majority of studies in Lepidoptera have been in two moth species; the cabbage looper,*Trichoplusia ni* (Noctuidae) and the tobacco hornworm, *Manduca sexta* (Sphingidae). As such, references to Lepidoptera in this thesis generally refer to moths, in particular *T. ni* and *M. sexta*.

1.1 Characterisation of JH Hydrolytic Enzymes

JHE has been isolated from a number of insect species, mainly moths and most of these JHE forms are monomers with a molecular weight of around 60kD (Coudron *et al.*, 1981; Rudnicka and Kochman, 1984; Abdel-Aal and Hammock, 1986; 1988; Rudnicka and Jones, 1987; Valaitis, 1991; 1992). JHE is a carboxylesterase containing sequences with high levels of conservation around features identifying it as a member of the carboxyl/choline esterase multigene family. JHE is believed to have a structural feature common with other members of this family, the "α/β hydrolase fold" (Ollis *et al.*, 1992). Enzymes with the "α/β hydrolase fold" commonly contain a nucleophile, a histidine and an acid (Wojtasek and Prestwich, 1996). In *Heliothis virescens*, JHE contains such a catalytic triad (Ser201, Glu332 and His446) which hydrolyses its substrate, JH (Ward *et al.*, 1992).

JHE from *D. melanogaster* is a monomeric 66 kDa protein. The JHE protein isolated from *D. melanogaster* pupae is believed to have a serine active site based on its sensitivity to inhibitors (see section 1.1.1; Campbell *et al.*, 1992). Seven amino acids have been sequenced from the N-terminus of the protein and no sequence similarity to other sequenced JHE proteins was evident (Campbell *et al.*, 1998). While this is the only form of JHE believed to exist in *D. melanogaster*, in *D. virilis* two forms of JHE are believed to exist (Rauschenbach *et al.*, 1995; Campbell *et al.*, 1992; 1998).

Much less is known about JHEH enzymes in insects and their physiological role is less well understood than that of JHE. JHEHs have been partially characterised in *D. melanogaster, M. sexta* and *Galleria mellonella* (Share *et al.,* 1988; Harshman *et al.,* 1991; Casas *et al.,* 1991; Jesudason *et al.*, 1992; Wojtasek and Prestwich, 1996). The gene encoding one JHEH, from *M. sexta,* has been isolated. Sequence analysis suggests that this JHEH belongs to the same family as JHE; the α/β hydrolase fold family (Pinot *et al.*, 1995; Wojtasek and Prestwich, 1996).

1.1.1 Inhibitor Sensitivity

The active sites of lepidopteran JHE enzymes were originally characterised by sensitivity to inhibitors. The original definition for a JH-specific esterase was based on studies on JHEs in haemolymph of lepidopteran larvae which generally were resistant to inhibition with the organophosphate di-isopropylphosphofluoridate (DFP; Sanburg *et al.,* 1975). DFP inhibits enzyme activity by binding to the active site serine in carboxylesterases (Sanburg *et al.,* 1975). However, JHE enzymes from the flesh fly, *Sarcophaga bullata*, the blowfly, *Phormia regina* and *D. melanogaster* are sensitive to DFP (Yu and Terriere, 1978a; Campbell *et al.,* 1992), although JHEs from other Diptera, such as the house fly, *Musca domestica,* are resistant (Sparks and Hammock, 1980). *D. virilis* is unusual in that it has two forms of JHE, one sensitive to DFP (believed to be the homologue of the *D. melanogaster* enzyme) and one resistant (Rauschenbauch *et al.,* 1995; 1996). Other enzyme inhibitors, such as 3-(octylthio)-1,1,1-trifluoropropan-2-one (OTFP), are highly specific for JHEs in Lepidoptera and also inhibit JHE in *D. melanogaster* and *D. virilis* (Hammock, 1985; Campbell *et al.,* 1992; Rauschenbauch *et al.,* 1995; 1996). Subsequently, the inhibitor sensitivities of JHE enzymes from several species of Diptera were not consistent with the original definition of JH-specific esterases being resistant to DFP, suggesting that the structure of JHE in Diptera and Lepidoptera may be different (Campbell *et al.,* 1992).

No inhibitors of JHEH activity have yet been found despite an extensive search in *D. melanogaster*, the blowfly, *Calliphora erythrocephela,* and various species of moth (Brooks, 1973; Slade *et al.,* 1975; Yu and Terriere, 1978b; Mullin and Wilkinson, 1980; Harshman *et al.,* 1991).

1.1.2 Isoforms of JH Hydrolysing Enzymes

Until recently there has been limited evidence for multiple JHE isoforms in Diptera, although in Lepidoptera isoforms are commonly reported (Kramer and de Kort, 1976; Rudnicka and Kochman, 1984; Woodring and Hoffman, 1997). The number of JHE isoforms found in different lepidopteran species varies greatly. JHE isoforms are often tissue specific and in the case of *M. sexta*, where ten isoforms have been detected, unique JHE isoforms are found in every tissue studied (Jesudason *et al.,* 1992). The molecular basis for JHE isoforms is believed not to be multiple genes but rather allelism or post-translational modifications, such as glycosylation (D. Jones *et al.,* 1986; Jones and Click, 1987; Hanzlik and Hammock, 1987; Abdel-Aal and Hammock, 1988; Share *et al.,* 1988; Hanzlik *et al.,* 1989; Roe and Venkatesh, 1990).

In *D. melanogaster,* evidence for multiple forms of JHE is circumstantial. Isoelectric focusing of the JHE protein from *D. melanogaster* prepupae showed no evidence of multiple JHE isoforms and during the purification of JHE from prepupae a single peak of JHE activity was recovered from each purification step (Campbell *et al.,* 1992). However, in adults, JHE activity is associated with different subcellular fractions and activity shows differential stability after freezing (Campbell *et al.,* 1992). These observations suggest that more than one form of JHE activity may exist in *D. melanogaster.*

In other fly species, recent evidence suggests that multiple forms of JHE exist. In *D. virilis*, the two forms of JHE were present in pupal and adult life stages, although one of these forms is thought to change its conformation in the adult stage (Rauschenbauch *et al.,* 1995). In the

mosquito, *Culex quinquefasciatus,* three stage-specific JHE isoforms were detected in the cytosolic fraction of 36 hour fourth-instar larvae and pharate pupae (Lassiter *et al.*, 1995). Two of these isoforms were detected tissue-specifically in adults and it has been suggested that the two isoforms represent functional variants of JHE (Lassiter *et al.*, 1996). In *M. domestica*, there are soluble and membrane associated forms of JHE with different stabilities and inhibitor sensitivities suggesting that multiple forms of JHE exist (Sparks and Hammock, 1980).

Multiple JHEH isoforms are also believed to exist in lepidopterans, including *M. sexta* (Casas *et al.,* 1991; Jesudason *et al.,* 1992) and dipterans, including *D. melanogaster* (Harshman *et al.,* 1991). In the mosquito, *C. quinquefasciatus,* six different stage-specific JHEH isoforms were detected on isoelectric focusing gels (Lassiter *et al.,* 1995).

1.1.3 Characterisation of Genes Encoding JHE and JHEH

The JHE gene was first isolated and characterised in *H. virescens* (Hanzlik *et al.,* 1989; Harshman *et al.*, 1994). Subsequently, the gene was isolated and characterised in *T. ni* and more recently a cDNA clone has been isolated in the Colorado potato beetle *Liptinotarsa decemlineata* (Venkataraman *et al.,* 1994; Schelling and Jones, 1995; Vermunt *et al.,* 1997). JHE genes in *H. virescens* and *T. ni*, appear to exist as single-copies which produce single mRNA species (Hanzlik *et al.,* 1989; Wroblewski *et al.,* 1990; Harshman *et al.,* 1994).

In *T. ni*, 1.2Kb of genomic DNA upstream of the JHE coding sequences has been sequenced and a transcription start site, a TATA box and a core promoter characterised (Venkataraman *et al.,* 1994; Jones *et al.*, 1998). Several sequences have been identified that are similar to or resemble consensus elements that may have regulatory significance, including a possible JH regulatory site. Results so far indicate that regulation of the JHE gene is transcriptionally mediated through interaction of nuclear regulatory proteins with sequence elements in the promoter.

In *T. ni*, RNA synthesis from the JHE gene is induced by at least two different transcriptional activators, a neurosecretory protein from the brain and JH (Venkataraman *et al.*, 1994). Although little is known of the neurosecretory protein, synthesis of the JHE transcript in prepupae is induced by JH alone and no other hormonal influence is necessary (Venkataraman *et al.*, 1994). It is also apparent that JH may act at other levels of JHE regulation, including stability of the message and regulation of the rate of secretion of JHE out of the fat body (Venkataraman *et al.*, 1994).

Isolation of the JHE gene from Diptera has to date been unsuccessful. However, the cDNA of *H. virescens* JHE does not hybridize with *D. melanogaster* genomic DNA and antibodies raised against *H. virescens* JHE do not cross react with *D. melanogaster* JHE (S. Fieg, M. Healy and J. Oakeshott, unpublished). Mating studies in *D. virilis* suggest that JHE activity is under the control of a single gene (Rauschenbach *et al.*, 1995).

A cDNA clone encoding JHEH has been isolated from *M. sexta* eggs. Sequence analysis of this microsomal epoxide hydrolase shows a high level of sequence similarity to mammalian microsomal epoxide hydrolases suggesting that these enzymes are derived from a common ancestral gene (Wojtasek and Prestwich, 1996).

1.2 Patterns of Expression of JH Hydrolytic Enzymes

1.2.1 Developmental Profile

JH hydrolysis has been studied extensively in several species of Lepidoptera, particularly in the last larval instar. Generally, two distinct peaks of activity are defined in the last larval instar; one at the prewandering stage and a second at the prepupal stage. The prewandering peak of activity is believed to induce wandering behaviour and commitment to pupation while the prepupal peak, which occurs immediately prior to pupation, is required for ecdysis

(Jones, 1985; Jones and Hammock, 1985). Both peaks follow a decrease in JH synthesis and a subsequent decline in the titre of circulating JH. In Diptera, major peaks of JHE and JHEH activities are present in prewandering larvae and prepupae and these peaks also follow declines in JH synthesis and whole body JH titre (Bownes and Rembold, 1987; Sliter *et al.*, 1987; Richard *et al.*, 1989a; 1989b; Campbell *et al.*, 1992). These peaks in JH hydrolytic activity have been identified in several species of Diptera, including *D. melanogaster*, *D. virilis* and *C. quinquefasciatus*.

The relative contributions of JHE and JHEH to total JH hydrolysis in last instar larvae differ considerably between species. Similar profiles of JH hydrolysis are found in *D. melanogaster* and *T. ni,* where the prewandering peak of JH hydrolysis is due mainly to JHEH activity and the prepupal peak is due mainly to JHE activity (Campbell *et al.*, 1992; Kallapur *et al.*, 1996; Khlebodarova *et al.*, 1996). In contrast, both peaks in *C. quinquefasciatus* and *M. sexta* are primarily due to JHEH activity. However, in *C. quinquefasciatus,* JHE activity is minimal in the final instar while in *M. sexta* JHE activity makes a relatively substantial contribution to JH hydrolysis and is regulated independently of JHEH activity (Jesudason *et al.*, 1992; Lassiter *et al.*, 1995).

Hydrolysis of JH in adult Lepidoptera and Diptera has not been well studied. In *D. melanogaster,* relatively low levels of both JHE and JHEH specific activities are maintained in virgin adults and both enzymes contributed approximately equally to JH hydrolysis (Campbell *et al.*, 1992). However, another study on JH hydrolysis in adult *D. melanogaster,* measured activity per individual rather than specific activity and found that JHEH activity rises to relatively high levels during adult maturation while JHE activity declines (Khleboderova *et al.*, 1996). In the same study, JH hydrolysis in *D. virilis* was also measured. In adults, JH hydrolysis was predominantly due to JHE activity which rose quickly at eclosion and continued to maintain a high titre for at least five days (Khleboderova *et al.*, 1996).

1.2.2 Spatial Profile

1.2.2.1 Tissue Localisation

Extensive spatial analyses of JHE and JHEH activities have been carried out in a few species of Lepidoptera, including the larvae of *M. sexta* and *T. ni.* In both species, peaks of JH hydrolysis are present in tissues isolated at the prewandering and prepupal stages. In *T. ni*, both JHE and JHEH activities are present in integument, midgut and fatbody and in *M. sexta*, both activities are present in integument, midgut, Malphighian tubules, fatbody and brain (Jesudason *et al.*, 1992; Grieneisen *et al.*, 1995; Kallapur *et al.*, 1996). In the haemolymph of both species, only JHE activity has been detected which is believed to be secreted from the fat body (Sparks and Hammock, 1979; Wing *et al.*, 1981; Jones and Click, 1987; Jesudason *et al.*, 1992; Kallapur *et al.*, 1996).

Few detailed studies on JH hydrolysis in tissues have been undertaken in Diptera. Most of the early studies focused on JH hydrolysis in the fat body and haemolymph of larval and pupal life stages (Klages and Emmerich, 1979; Bisser and Emmerich, 1981; Shapiro *et al.*, 1986). In *D. hydei* larvae, the fat body contained high levels of JHE and JHEH activities and the body wall had high levels of JHE activity (Klages and Emmerich, 1979). The haemolymph of *D. hydei* larvae had no JH hydrolytic activity while in prepupae and pupae, haemolymph and body fluid contained relatively high levels of JHE activity (Klages and Emmerich, 1979; Bisser and Emmerich, 1981). In *D. melanogaster*, JH hydrolysis in haemolymph of last instar larvae and adults was very low (Wilson and Gilbert, 1978). In the mosquito, *Aedes aegypti*, both the ovary and the fat body produced similar levels of JHE in organ culture and blood-fed adults had much higher levels of activity than unfed adults (Shapiro *et al.*, 1986).

The spatial distribution of JH hydrolysing enzymes in the Diptera has been studied most extensively in *C. quinquefasciatus.* In this species, the distribution of JHE and JHEH

activities have been examined in females immediately following a blood meal (Lassiter *et al.*, 1996). JHE and JHEH were present in all tissues that have been examined, including ovaries, gut, head and carcass. JHEH activity was considerably higher than JHE activity in all tissues and both enzymes were concentrated in the gut. There was tissue-specific variation in the time of peak enzyme activity following a blood meal and the activity of the enzymes peaked independently. For example, JHE activity peaked in the head twelve hours earlier than in the other tissues while JHEH activity in the ovary declined after the blood meal but increased in all other tissues (Lassiter *et al.*, 1996)

One major difference between the Lepidoptera and Diptera, in the spatial profiles of JH hydrolytic enzymes, is in the level of JH hydrolysis in the haemolymph. The species of Diptera examined generally had either very low levels or no JH hydrolysis in haemolymph but in the species of Lepidoptera studied, relatively high levels of JHE activity were present in haemolymph (Roe and Venkatesh, 1990). In contrast, both Diptera and Lepidoptera had high levels of JH hydrolysis in digestive systems (Kallapur *et al.*, 1996; Lassiter *et al.*, 1996). In last instar larvae of *M. sexta*, JH hydrolysis has been localised to the Malphighian tubules and midguts (Jesudason *et al.*, 1992; Grieneisen *et al.*, 1995). Both these tissues are specialised for processing and removal of compounds from the insect circulatory system (Jesudason *et al.*, 1992).

1.2.2.2 Subcellular Localisation

JHE activity in Lepidoptera is generally cytosolic although analysis of individual tissues from *M. sexta* revealed some differences in localisation (Roe and Venkatesh, 1990; Jesudason *et al.*, 1992). Very small amounts of JHE activity were present in membrane-bound fractions of the midgut and fat body. However, in the integument only one-fifth of the JHE activity was membrane-bound and in the brain and haemolymph, all JHE activity was cytosolic (Jesudason *et al.*, 1992). In contrast, the majority of JHEH activity in most

lepidopterans is membrane-bound (Roe and Venkatesh, 1990); in *M. sexta*, 60-80% of JHEH activity in all tissues studied was membrane-bound (Jesudason *et al.*, 1992).

In Diptera, subcellular localisation of JH hydrolytic activity has varied considerably among species. In *D. melanogaster,* JH hydrolytic activity in prewandering larvae and adults is mainly JHEH activity associated with membrane-bound fractions. In prewandering larvae, the majority of JHEH activity is associated with mitochondria and a smaller amount is in the microsomal fraction. In adults, the JHEH activity is also mainly in the mitochondrial fraction while the JHE activity is distributed equally between the microsomal and soluble fractions (Ottea *et al.*, 1988; Casas *et al.*, 1991; Harshman *et al.*, 1991; Campbell *et al.*, 1992). In prepupae and pupae, JH hydrolysis is due to JHE activity in soluble fractions (Ottea *et al.*, 1988; Campbell *et al.*, 1992).

Studies on other dipteran species show different subcellular distributions. In adult *M. domestica*, both JHE and JHEH activities are predominantly microsomal and in *C. quinquefasciatus* larvae and pupae both JHEH and JHE activities are predominantly membrane-bound (Yu and Terriere, 1978b; Lassiter *et al.*, 1995).

1.3 Biological Roles of JH

1.3.1 Roles in Larvae/Pupae

The best characterised role for JH in insects is determining the nature of the larval moult by modifying molecular, cellular and organismal responses to the moulting hormone 20-hydroxyecdysone (Riddiford, 1994; Restifo and Wilson, 1998). In the last larval instar, JH is believed to maintain larval-specific organs and also may be involved in maintenance of larval pigment proteins, feeding behaviour and the high level of metabolism found in this lifestage (Riddiford, 1994). In premetamorphic Lepidoptera, JH appears to be important for normal dorsal closure, formation of the larval cuticle and differentiation of the midgut. In

some insects, JH may be important for the production of prothoracicotropic hormone (PTTH; Riddiford, 1994). The role of JH in the development of prepupae varies among species of Lepidoptera (Riddiford, 1994). For example, JH is believed to be essential for stimulating ecdysone secretion and subsequently pupation in *T. ni* (Jones and Hammock, 1985). However, JH is believed to suppress precocious adult development in *M. sexta* (Kiguchi and Riddiford, 1978; Roe and Venkatesh, 1990).

The roles of JH in the larval life stage of any species of the Diptera are not as well characterised. Commitment of the larval cells to metamorphose occurs at different times in Diptera and Lepidoptera, suggesting that the specific involvement of JH in larval and pupal development in these orders differ. JH in Diptera may allow the imaginal discs to proliferate after they attain competence to metamorphose in the second instar since JH prevents normal metamorphosis of mature larval discs in response to 20-hydroxyecdysone *in vitro* (Doctor and Fristrom, 1985; Riddiford and Ashburner, 1991). Evidence of JH binding proteins in the integument of third-instar larvae of *D. hydei,* as well as haemolymph and fat body of third-instar larvae *D. melanogaster,* also suggests that JH may play some role in these tissues (Klages *et al.*, 1980; Shemshedini and Wilson, 1988; 1993).

1.3.2 Roles in Adults

In adult Diptera, the role of JH is believed to vary according to the life style and the impact of nutritional variation on the neuroendocrine system (Yin and Stoffolano, 1997). As such, it is difficult to make generalisations about the impact of JH on adult insects, in particular on reproductive processes. In *D. melanogaster,* JH is believed to play a number of roles in both female and male reproductive processes and in both sexes, JH binding proteins have been identified (Wyatt and Davey, 1996). In females, JH influences vitellogenesis in the fat body and the development of eggs (Bownes and Reid, 1990). JH is believed to induce the follicle cells to produce ecdysteroids and to prime the fat body to respond to 20-hydroxyecdysone. Ecdysteroids stimulate vitellogenesis in the fat body and allow the follicle cells to be

responsive to egg development neurosecretory hormone (Schwartz *et al.,* 1985; 1989; Bownes, 1989). JH is also believed to be involved in receptor-mediated endocytosis in ovaries, whereby vitellogenin is taken up by the oocytes (Tedesco *et al.*,1981; Wyatt and Davey, 1996). Yolk protein synthesis and the uptake of these yolk proteins into the oocytes is stimulated by JH treatment (Gavin and Williamson, 1976; Jowett and Postlethwait, 1980; Postlethwait and Shirk, 1981; Koeppe *et al.,* 1985).

In other insects, JH has also been implicated in patency; the creation of space between the follicle cells. In *Rhodnius*, JH is believed to act via membrane receptors and possibly a nuclear receptor within the follicle cells (Davey, 1981; Ilenchuk and Davey, 1987a; 1987b). A similar system operates in the patency of *Locusta migratoria* and a membrane associated JH binding protein has been isolated (Wyatt and Davey, 1996).

In male insects, JH has also been implicated with processes in the reproductive system, in particular in the accessory glands, the testes and the spermathecae. In some insects, JH stimulates the accessory glands to take up proteins from the haemolymph and may also play a role in the accumulation and secretion of proteins in the accessory glands (Wyatt and Davey, 1996).

In *D. melanogaster*, a number of experiments have implicated JH in the accumulation of proteins in the accessory glands. These include observations that in the *Met* mutant of *D. melanogaster*, which has a reduced JH response, there is lower total protein accumulation in accessory glands than in control glands (Shemshedini and Wilson, 1990). In addition, JH treatment of transgenic *D. melanogaster* males, which were carrying a marker gene driven by the promoter from an accessory gland gene, stimulated protein synthesis of the marker gene. The accessory gland gene from which the promoter was derived is known to secreted and transferred to the female during mating. This JH effect is the same as that observed for accessory gland proteins after mating suggesting that JH may play a role in mating induced synthesis of accessory gland proteins (Herndon *et al.,* 1997). JH, in presence of calcium, has

also been observed to stimulate both protein and RNA synthesis nearly three-fold in the accessory glands of *D. melanogaster* males (Yamamoto *et al.*, 1988; Shemshedini and Wilson, 1993). It is possible that JH has two sites of action, the cytoplasm and the nucleus and it acts via a membrane receptor and a nuclear receptor (Yamamoto *et al.*, 1988). JH binding proteins have been isolated from both the cytosolic and nuclear fractions of accessory glands from *D. melanogaster,* also suggesting that JH has different modes of action (Shemshedini and Wilson, 1990).

JH also influences certain processes in spermathecae and has an indirect role in the testes of some insects, including effects on spermatogenesis and influence on the early appearance of mature sperm (Wyatt and Davey, 1996). JH is also thought to affect responsiveness to sex pheromone in Lepidoptera and to stimulate the synthesis of pheromone components (Gadenne, 1993; Wicker and Jallon, 1995; Wyatt and Davey, 1996).

1.4 Biological Roles of JHE and JHEH

One role of JHE and JHEH activities in insects, is to regulate the titre of JH during development (Roe and Venkatesh, 1990; Riddiford, 1994). However, differences in the specific roles of these enzymes in insects may occur because of the different developmental patterns of expression and different pathways of JH biosynthesis.

1.4.1 Roles in Larvae/Pupae

In Lepidoptera, there is a peak of JHE activity toward the end of each larval instar. The function of this peak in JHE activity is unknown but it is generally believed that it is necessary for regulation of low levels of JH (Jones and Click, 1987; Roe and Venkatesh, 1990; Riddiford, 1994). However, treatment of larvae with JHE inhibitors or JH have no detectable effect on larval development (Jones and Click, 1987).

In prewandering lepidopterans, a small peak of ecdysone, almost coincident with the peak in JH hydrolysis, is responsible for commitment to pupation (Roe and Venkatesh, 1990; Riddiford, 1994). Small amounts of JH can inhibit the production and release of ecdysteroids and subsequently, commitment to pupation. Wandering behaviour can be delayed and the phenomenon of supernumerary moults may occur, whereby the larvae undergo extra instars which do not survive (Cymborowski *et al.*, 1982; Jones and Click, 1987; Roe and Venkatesh, 1990; Riddiford, 1994). Increasing the titre of JH at this time, either by inhibition of JHE activity or by topical application of JH, causes a delay in ecdysone release and wandering behaviour, production of larval-pupal and pupal-adult intermediates and a block in pupation (Jones, 1985; Jones and Hammock, 1985; Jones and Click, 1987; Sparks *et al.*, 1987; Jones *et al.*, 1990). Finally, JHE has a role in commitment of last instar lepidopteran larvae to metamorphosis by removing all traces of JH from circulation (Jones *et al.*, 1990; Roe and Venkatesh, 1990).

In prepupal Lepidoptera, a second peak of JH hydrolysis occurs following peaks in ecdysone and JH. While the ecdysone levels remain high throughout pupation, the JH titre decreases (Roe and Venkatesh, 1990; Riddiford, 1994). Treatment of *T. ni* prepupae with JHE inhibitors or JH to increase JH titre, disrupts pupation and ecdysis by producing intermediate forms (Jones and Hammock, 1983; Jones and Hammock, 1985; Jones *et al.*, 1986; Roe and Venkatesh, 1990; Riddiford, 1994). It has been suggested that artificially increasing the JH titre at this time blocks the release of eclosion hormone by indirectly increasing the titre of ecdysone (Truman *et al.*, 1981). Thus, evidence suggests that the role of JH hydrolysis at this lifestage is also to remove the final traces of JH to allow metamorphosis to occur (Roe and Venkatesh, 1990).

In Diptera, peaks of ecdysone are observed in the final larval instar similar to those observed in Lepidoptera although the function of these peaks is unknown (Roe and Venkatesh, 1990). In *D. melanogaster*, continual exposure to JH throughout larval life does not affect development until the final instar when the differentiation of adult structures occurs (Roe and

Venkatesh, 1990). Exposure to a low concentration of JH throughout larval life is enough to disrupt adult abdominal development and subsequent eclosion. However, high concentrations of JH are needed to prevent normal adult differentiation of imaginal discs (Riddiford and Ashburner, 1991).

The theory explaining these unique effects of JH on development in Lepidoptera and higher Diptera is based on the timing of imaginal disc differentiation and commitment to metamorphosis (Riddiford and Ashburner, 1991; Campbell *et al.*, 1992). In *D. melanogaster*, the imaginal discs begin differentiating to adult structures early in the first instar and most are committed to metamorphosis some time in the second instar while the abdominal histoblasts do not differentiate until after pupariation (Madhaven and Schneiderman, 1977; Roseland and Schneiderman, 1979; Riddiford and Ashburner, 1991). In contrast, the imaginal discs in lepidopteran larvae do not begin to differentiate and become committed to metamorphosis until the end of the last larval instar (Kiguchi and Riddiford, 1978). Subsequently, JH treatment has more pronounced effects on imaginal disc differentiation in the final instar of lepidopteran species compared to dipteran species. However, presumably the role of JHE and JHEH activities in dipterans is also to remove the final traces of JH from circulation as they do in lepidopterans (Campbell *et al.*, 1992). Additional evidence that JHE activity is needed for metamorphosis to occur normally is that treatment of *D. virilis* wandering larvae or white prepupae with a JHE inhibitor, paraoxon, results in inhibition of the normal prepupal peak of JH hydrolysis and subsequent pupal death (Rauschenbach *et al.*, 1991).

Finally, it has been suggested that the primary role of JHEH in Lepidoptera is to metabolise JH acid to the inactive JH acid-diol. JH acid is an intermediate in JH biosynthesis and has hormonal effects in certain JH target tissues (Sparagana *et al.*, 1985; Ismail *et al.*, 1998). JH acid is also produced by the hydrolysis of JH by JHE and can subsequently be recycled back to JH in particular organs, such as imaginal discs and male accessory glands (Sparagana *et al.*, 1985). In Diptera, recycling of JH does not occur and JH acid is not known to have a

biological role. Also, the production of JH acid-diol has not been observed in *D. melanogaster,* at least after *in vitro* hydrolysis of JHIII (Campbell *et al.,* 1992). Subsequently, the role of JHEH in Diptera does not appear to involved the metabolism of JH acid.

1.4.2 Roles in Adults

The role of JH hydrolysis in adult Diptera has been most studied with respect to female reproduction. JH hydrolysis may play a role in ensuring that low JH titres are maintained as is necessary for certain stages of reproductive development (Khlebodarova *et al.,* 1996). In *Drosophila*, there is continuous maturation and oviposition of eggs, each step of this process requiring different levels of JH (Koeppe *et al.,* 1985; Bownes, 1989; Wyatt and Davey, 1996). It has therefore been argued that localised titres of JH may exist in different organs of the *Drosophila* reproductive system (Khlebodarova *et al.,* 1996). This may be achieved through intense synthesis and rapid degradation of JH. In this model, JH hydrolysis must play a crucial role in *Drosophila* reproduction. It was shown by using a mutant *D. virilis* line, characterised by lower levels of JH degradation compared to wildtype, that delayed increases in JHE activity in female adults also delayed oviposition. Also, injection of JHE into the mutant females allowed precocious oviposition to occur (Khlebodarova *et al.,* 1996).

Studies to examine the relationship between the inhibitory effects of OTFP (an inhibitor of JHE activity) on JH hydrolysis, oviposition and fertility in *D. melanogaster* and *D. virilis* revealed that both JHE and JHEH activities influenced fertility in both species. In *D. virilis,* JHE activity played the more significant role in oviposition (Rauschenbach *et al.,* 1996).

Correlative evidence suggests that JH hydrolysis also plays a role in mosquito reproduction. In female *A. aegypti*, a brief decrease in JH biosynthesis and titre is believed to initiate vitellogenesis and allow normal egg development (Shapiro *et al.,* 1986; Borovsky *et al.,* 1992). The decline in JH titre corresponds with a peak in JHE activity (Shapiro *et al.,* 1986).

Similar observations have been reported in other species of mosquito (Lassiter *et al.*, 1996). It was suggested that the peak in JHE activity contributes to regulation of JH titre by scavenging the final traces of JH, which allows normal egg development to resume (Shapiro *et al.*, 1986). Treatment of *A. aegyti* females with a JHE inhibitor caused a reduction in egg hatch number, supporting this theory (Shapiro *et al.*, 1986).

Although limited studies on adult Lepidoptera have been reported, it is known that female *T. ni* adults require high levels of JH for successful maturation of eggs and oviposition (Venkatesh and Roe, 1988). JHE activity declines to low levels in mated *T. ni* females and remains high in virgin females, which have reduced oviposition and retain undeveloped eggs until mating (Venkatesh and Roe, 1988). High JHE activity in virgin females might maintain a low level of JH causing reproductive development to be arrested (Venkatesh and Roe, 1988). This theory was tested by injecting recombinant JHE, derived from a lepidopteran, into the cricket, *Acheta domesticus*, which relies on high levels of JH for normal development of eggs, as do lepidopterans. The outcome was a decrease in egg production and a slowing down in ovarian development consistent with the theory (Bonning *et al.*, 1997).

1.5 Regulation of JH

JH biosynthesis occurs in a specialised endocrine gland, the corpora allata which is extensively innervated in all insects (Feyereisen, 1985; Giebultowitz and Denlinger, 1985). In larvae of higher Diptera, the corpora allata consists of a compact cluster of cells called the ring gland, which includes the corpora allata, corpus cardiacum and the prothoracic glands (King *et al.*, 1966; Meurant and Sernia, 1993). JH biosynthesis has also been found to occur in other organs of the body including accessory glands of male mosquitoes and the ovaries of female mosquitoes (Borovsky *et al.*, 1994a; 1994b). The regulation of JH biosynthesis involves a number of steps and no single rate-limiting step has been found. Studies suggest that the brain is vital to the regulation of corpora allata activity by either having a stimulatory

or inhibitory effect (Altaratz *et al.*, 1991; Richard and Gilbert, 1991). The brain can act on the corpora allata either neurally, hormonally or by a combination of both (Sehnal and Granger, 1975; Bhaskaran and Jones, 1980; Couillaud *et al.*, 1984; Bhaskaran *et al.*, 1990; Richard *et al.*, 1990; Lourdes *et al.*, 1991; Duve *et al.*, 1992). Other factors also affect the corpora allata in some insect species, including factors released from the ovary (Feyereisen, 1985). In *H. virescens*, mating is believed to stimulate JH biosynthesis in the female corpora allata (Park *et al.*, 1998). In two species of cricket, JHE activity released from the adult female corpora cardiaca (a structure connected to the corpora allata) is believed to cause a decrease in the biosynthesis of JH by the corpora allata (Woodring and Hoffman, 1997). In the few insect species studied, the rate of release of JH from the corpora allata is proportional to the rate of synthesis suggesting that the JH release is not regulated (Tobe and Pratt, 1974).

The availability of circulating JH is believed to be regulated by juvenile hormone binding proteins (JHBP) in the form of lipophorins (Trowell, 1992). Lipophorins are found in all insects and have been detected in vitellogenic ovaries from a number of insects, including the flesh fly, *S. bullata* (Van Mellaert *et al.*, 1985; Trowell, 1992). The most recent model to explain the interaction of JHE and JHEH activities with JH in lepidopterans involves a JHBP. It has been suggested that JH is protected by a JHBP from degradation by JHEH and non-specific enzymes. However, in some situations JHBP may facilitate in the degradation of JH by JHE. At low concentrations of JHE the rate of JH degradation, predicted using kinetic constants for the isolated JH protein, is similar to experimental observations. This suggests that JHBP does not influence JH hydrolysis by JHE. However, at high concentrations of JHE the degradation of JH is much greater than would be predicted by these constants, suggesting that JHBP is in fact assisting in the clearance of JH (Touhara *et al.*, 1995).

JHBP is believed to facilitate the degradation of JH in several ways. First, JH is removed from lipophilic deposits thus returning it to circulation and the possibility of degradation. Second, in the presence of high concentrations of JHE, JHBP is believed to present the JH in a position which increases the efficiency of hydrolysis by JHE but not by other enzymes,

including JHEH. After the JH is cleaved the resulting JH acid may then enter the tissues where JHEH and conjugation reactions degrade the JH acid to an irreversibly inactivated molecule (Touhara *et al.*, 1995).

Recently, in *D. melanogaster,* it was observed that the rate of hydrolysis of high concentrations of JH by JHE is unaffected by the presence of dipteran lipophorin. However, at lower concentrations of JH, hydrolysis by JHE is reduced in the presence of lipophorin (Campbell *et al.*, 1998). It was calculated that lipophorin inhibits JH hydrolysis by simply lowering the concentration of unbound JH available to JHE (Campbell *et al.*, 1998). Low concentrations of dipteran lipophorin are also believed to remove JH from inaccessible binding sites keeping it in circulation and available for hydrolysis. At higher concentration of lipophorin, competition for JH binding sites may affect JH hydrolysis (Trowell, 1992; Trowell *et al.*, 1994).

1.6 Regulation of JHE and JHEH

JHE and JHEH activities are regulated at many levels in insects. These include developmental, spatial, sex-specific, isoform-specific and hormone-specific regulation. JHE and JHEH are also regulated independently of each other i.e. differentially, in most insects. The factors regulating the patterns of expression of JHE and JHEH activities have been well studied in some insects. Although most of the factors that regulate these patterns of expression are not well known it is evident that more than one factor is involved (Hammock, 1985; Roe and Venkatesh, 1990). There are many examples of JHE and JHEH activities being regulated at different levels in the Diptera.

In *D. melanogaster* and *D. virilis*, JHE and JHEH activities are regulated developmentally, sex-specifically and differentially (Campbell *et al.*, 1992; Rauschenbach *et al.*, 1995; Khlebodarova *et al.*, 1996). In *D. virilis,* the two forms of JHE are synchronously regulated from larval-pupal development to adult emergence. However, after emergence the activity of

the DFP-insensitive form of JHE increases while the activity of the sensitive form does not (Khlebodarova *et al.*, 1996). Furthermore, regulation of JHE activity in adults is sex-specific as evidenced by the decrease in activity in females but not in males under heat stress (Rauschenbach *et al.*, 1995).

In *C. quinquefasciatus*, the JHE isoforms present are differentially, developmentally and tissue-specifically regulated and all JHE isoforms detected in the adult and larval stages have a tissue-specific distribution (Lassiter *et al.*, 1996). JHEH is also regulated independently of JHE in specific adult tissues. After a blood meal JHE and JHEH activities increase by different amounts in each tissue examined and peaks in activity occur at different times in each tissue and with each enzyme. It has been suggested that JH hydrolysing enzymes are subjected to tissue specific regulation because JH is synthesised in a tissue-specific manner (Lassiter *et al.*, 1996).

1.6.1 Regulation by JH

One of best studied factors known to regulate JHE activity is JH. In *T. ni* and *H. virescens* prepupae, JH treatment increased transcription from the JHE gene. However, in *H. virescens,* the levels of JHE mRNA and the levels of JHE activity induced by JH were not correlated (Wroblewski *et al.*, 1990; Venkataraman *et al.*, 1994; Jones *et al.*, 1996). While JH regulates JHE primarily at the level of the gene it may also regulate the production or release of JHE at other levels such as translation, post-translational processing or secretion (Wroblewski *et al.*, 1990; Venkataraman *et al.*, 1994). Other factors in association with JH might also influence the expression pattern of JHE in particular tissues. These may be tissue-specific transcriptional activators such as promotor elements that regulate the rate of transcription, or sequences within the RNA transcript that influence mRNA processing or stability of the mature transcript in the cytoplasm (Wroblewski *et al.*, 1990; Jones *et al.*, 1996).

In Diptera, few studies have addressed the effects of JH on JHE activity. In *C. quinquefasciatus*, methoprene treatment had no effect on JHE activity in larvae/pupae but in adult females, methoprene treatment reduced JHE activity 24 hours after a blood meal but by 36 hours after the blood meal JHE activity was increased (Lassiter *et al.,* 1996).

Regulation of JHEH by JH has not been as extensively studied as the regulation of JHE by JH. In Lepidoptera, JHEH activity is generally believed not to be regulated by JH (Jesudason *et al.,* 1992; Kallapur *et al.,* 1996). In Diptera, regulation of JHEH activity has been best studied in *C. quinquefasciatus,* where it is believed to play a greater role than JHE activity in regulating JH titre. JHEH activity was unaffected by methoprene treatment in all adult and pupal stages of *C. quinquefasciatus* tested, but in the last larval instar, methoprene eliminated the normal peaks in JHEH activity while JHE activity was unaffected (Lassiter *et al.,* 1995; 1996).

1.6.2 Regulation by other factors

Several factors, other than JH, also regulate JHE activity. Firstly, examination of a baculovirus expressing JHE in infected larvae, indicates that JHE can be rapidly removed from circulation by uptake into pericardial cells thus altering the effectiveness of the enzyme in removing JH from circulation. This process is believed to be a specific receptor-mediated endocytosis and may also be the process that causes the rapid removal of JHE at critical times in development (Ichinose *et al.,* 1992; Booth *et al.*, 1992). Environmental factors such as food availability or starvation, stress due to temperature or injury, nutritional factors and parasitism may influence JHE activity indirectly, particularly via changes in JH titre (Roe and Venkatesh, 1990). In *D. virilis*, heat stress causes a decrease in JHE activity via a decrease in protein while in *T. ni,* heat stress induces the abundance of JHE transcripts (Rauschenbach *et al.,* 1995; Schelling and Jones, 1996).

Factors involved in the regulation of JHEH activity are not well studied, however, changes in activity due to exposure to particular chemicals have been reported. In *M. domestica*, adults exposed to phenobarbital induced JHEH activity by 78% (Yu and Terriere, 1978a). In *D. melanogaster*, adults exposed to cut orange for over 20 generations had increases in JHEH activity of up to 80% (Ottea *et al.*, 1988). These observations suggest that JHEH activity can be regulated but the nature of this regulation is unknown.

1.7 Aims and Outline of This Study

The major aims of this study on *D. melanogaster* were to:

1) determine the roles of JH hydrolytic enzymes by investigating the expression patterns of JH hydrolytic enzymes in juvenile and adult lifestages and,

2) determine the levels of regulation controlling JHE and JHEH activities, and in particular, investigate the regulatory relationship between JH and its hydrolytic enzymes in *D. melanogaster* compared with other Diptera and Lepidoptera.

Chapter two describes the temporal and spatial profiles of JHE and JHEH activities in last-instar larvae, prepupae and adult lifestages in *D. melanogaster.* These studies indicated the importance of degradation as a regulator of JH titre and the relative contributions of JHE and JHEH activities to JH hydrolysis. It also demonstrated that JHE and JHEH activities are regulated at a number of levels. A more detailed temporal profile from final instar larvae to pupae was compiled because in Lepidoptera, JHE is regulated by JH at this stage. Reports of the tissue distribution of JHE and JHEH activities in dipterans is scant. In this chapter, an extensive examination of the spatial distribution of JHE and JHEH activities in adults was undertaken. Comparisons of where JHE and JHEH activities are, in a range of tissues could then be made between *D. melanogaster* and other species of lepidopterans and dipterans.

Chapter three examines the role that JH plays in the regulation of JH hydrolytic enzymes, JHE and JHEH in *D. melanogaster,* at a number of lifestages; last instar larvae, prepupae and adults and in a range of adult tissues, mainly from the head and abdomen. In Lepidoptera, JH is believed to control its own hydrolysis by regulating the titre of JHE. JHEH does not appear to share such a close regulatory relationship with JH.

Chapter four discusses the distribution of JHE and JHEH activities in *D. melanogaster* and the possible roles that these activities may have and compares the roles with what is known in lepidopterans. The factors regulating JHE and JHEH activities are discussed and compared to lepidopterans.

Chapter Two

Temporal and Spatial Characterisation of JH Hydrolytic Enzymes

2.1 Introduction

Chapter two characterises the developmental and spatial profiles of JHE and JHEH activity in *D. melanogaster*. This information is then analysed to make predictions about the role of these enzymes and also how they are regulated.

In various species of Diptera and Lepidoptera, the relative contributions of JHE and JHEH activity to JH hydrolysis have been examined (Roe and Venkatesh, 1990; de Kort and Granger, 1996). Such studies have firstly elucidated the functional significance of JHE and JHEH activities i.e. to regulate the titre of JH, which is critical to normal development of the insect and secondly, determined the levels of regulation imposed on JHE and JHEH activities. In Lepidoptera and Diptera, these enzymes are generally found to be regulated at many levels (Roe and Venkatesh, 1990).

The temporal profiles of JHE and JHEH activities in *D. melanogaster* third-instar larvae to five day old adults, have previously been measured using relatively broad time points (Campbell *et al.*, 1992; Khleboderova *et al.*, 1996). There are two peaks of JH hydrolytic activity in larvae and pupae and a relatively constant level of JH hydrolytic activity in adults. To further investigate the relationship between JH hydrolytic enzymes and JH titre a more detailed JH hydrolysis profile was undertaken at three particular lifestages; at the time of peak JH hydrolysis in larvae and pupae, and at adult eclosion.

Extensive analysis of the spatial profiles of JH hydrolysing enzymes have been undertaken in one species of Diptera only, *C. quinquefasciatus* (Lassiter *et al.*, 1995) and two species of

Lepidoptera, *M. sexta* and *T. ni* (Jesudason *et al.*, 1992; Grieneisen *et al.*, 1995; Kallapur *et al.*, 1996). The study described in this chapter was undertaken to further understand the distribution of the JH hydrolytic enzymes in dipterans. The tissue ditribution of both JHE and JHEH was analysed in one day old *D. melanogaster* adults. Predictions about the localisation of JHE and JHEH activities in *D. melanogaster* adult tissues were made from reports of previous studies which indicated that the reproductive and digestive tissues in females and males, as well as the fat body in females are most likely to contain enzyme activities.

2.2 Materials and Methods

2.2.1 Drosophila Strains and Culturing Conditions

The *D. melanogaster* strain, 12Ill.2 (Cooke *et al.*, 1987) was used throughout this study. Organisms were cultured on agar-cornmeal-molasses media in 250ml plastic bottles at 25^0C, 65% humidity in a 12-12 hour light-dark cycle.

2.2.1.1. Temporal Profile

Larval age was determined using the following procedure: mated females were allowed to lay eggs on the standard media for four hours. About twenty-four hours later newly hatched first-instar larvae were selected by size and placed on fresh media at a density of 200 larvae per 12g media. It was assumed that these larvae had hatched within a four hour period of collection. Newly ecdysed third-instar larvae were collected at 72 hours after hatching and feeding third-instar larvae were collected every 5 or 10 hours from ecdysis until the wandering stage. Wandering occurred at about 105 hours until 115 hours after hatching. At each collection time point, 3-6 replicates of 30-50 larvae were collected, each replicate representing a different culture.

In general, pupal age was calculated using white prepupae (WPP) as the reference point. However, when greater accuracy was required prepupae and pupae were staged by the flotation method of Mitchell and Mitchell (1964). Moulting third-instar larvae were selected by observation of head eversion. Prepupal and pupal stages were collected every hour starting five hours after the white prepupal stage was reached. At each collection time point, 3-6 replicates of 7-15 prepupae/pupae were collected, each replicate representing a different culture.

Newly eclosed adults were collected as virgins and females and males aged separately. Adults were collected at 0, 1 and 2 days from the time of eclosion. At each time point adults were collected in three replicates of 5-10 individuals, each replicate representing a different culture.

All samples were frozen in liquid nitrogen and stored at -80^{0}C until homogenisation.

2.2.1.2 Tissue Profile

Tissues were collected from one day old adults and dissected in insect Ringer's solution. In the initial study adults were dissected and separated into head, thorax and abdomen. In the second study, brain and mouthparts were removed from the heads. The tissue remaining after removal of these organs was called the head carcass and included the eyes, antennae and cuticle. Three parts were dissected from abdomens 1) the reproductive system, which in females included the spermathecae, uterus and ovaries, and in males the testes, duct, bulb and accessory glands, 2) the digestive system which included the Malphigian tubules, midgut, intestine and rectum, and 3) the remaining tissue which was called the abdomen carcass and included the cuticle and fat body. The reproductive system was further dissected into individual tissues. In females, enzyme activity was assayed in two components 1) the spermathecae and uterus and 2) the ovaries. In males, three components were analysed for

enzyme activity 1) the testes 2) the duct and bulb and 3) the accessory glands. Haemolymph was extracted from the thorax. Each assay per tissue was replicated three times, each replicate from a different culture, consisting of tissues from 30 individuals. Whole organisms and dissected tissues were frozen in liquid nitrogen and stored at -80^{0}C.

2.2.2 JHE/JHEH Assays

All samples were homogenised in ice-cold 0.1M sodium phosphate buffer, pH 8.0. Homogenates were centrifuged at 15,600g for 10 minutes and the supernatants collected and stored at -80^{0}C.

JHE and JHEH activities were measured simultaneously and quantitatively using the radiometric partition assay of Hammock and Roe (1985) as modified by Campbell *et al.*, (1992). This assay was characterised for *D. melanogaster* by Campbell *et al.*, (1992). Their study determined qualitatively by thin layer chromatography, that JH acid and JH diol were the only products of the JH metabolism in *D. melanogaster* at three developmental stages; ninety hour larve, twelve hour pupae and five day old females. The only exception was in 12 hour pupae where there was a trace of an unidentified metabolite, in addition to JH acid and JH diol.

The study of Campbell *et al.*, (1992) also determined the partitioning of JH diol in the assay so that the separate activities of JHE and JHEH could be quantified. In this assay, it is known that JH acid partitions entirely into the aqueous phase, JHIII partitions entirely into the organic phase and JH diol partitions into both phases (Hammock and Roe, 1985). Campbell *et al.*, (1992) quantified the percentage of JH diol in the organic phase of a reaction: 56% of the JH diol partitions into the aqueous phase and 44% partitions into the organic phase.

A further modification to the radiometric partition assay in this study was that plastic eppendorf tubes were used throughout instead of glass tubes. Trial experiments indicate that this modification does not affect the final result of JH hydrolysis. The assay was undertaken as follows: 5-100µg of protein from homogenised tissue was diluted to 200µl with 0.1M sodium phosphate buffer, pH 8.0. Five µM (2µl) of a JHIII solution containing non-radiolabelled JHIII (Sigma) and between 0.06-0.08 pmoles of H^3 JHIII (13 000-16 000 cpm; NEN) was added to each diluted sample. (Stock solutions of JHIII were stored in hexane at -20^{0}C). The reaction was incubated at 30^{0}C for 30 minutes. JHE activity from the homogenate hydrolysed JHIII to JH acid while JHEH acitivity hydrolysed JHIII to JH diol. Hydrolysis of JHIII was stopped by mixing 100µl of the reaction with 25µl of a solution of methanol:water:ammonia (10:9:1). Isooctane (125µl) was added to the stopped reaction to remove unreacted JH from the aqueous phase. The remaining 100µl of the reaction was treated similarly; however the methanol: water:ammonia solution also contained 5mg/ml n-butane boronic acid (BBA) a derivatising agent for vicinal diols. BBA causes JH diol to partition entirely into the organic phase. As such, in the aliquot of the reaction mix containing BBA, the aqueous phase contained all the JH acid produced in the reaction and the organic phase contained all the JH diol produced. In the aliquot of the reaction mix which has no BBA, the aqueous phase contained all the JH acid produced plus 56% of the JH diol produced. One hundred µl of the aqueous phase of each half of the reaction was then added to 1ml OptiPhase 'HiSafe"LKB scintillation fluid and counted by liquid scintillation to determine the concentration of hydrolytic products.

For each batch of JHIII a series of test assays was undertaken to determine the linear range of the reaction. Test assays were done using tissue homogenates which were known to contain either high JHE or JHEH activities. Homogenates were assayed every 10 minutes for 60 minutes to determine the linearity of the time course of hydrolysis. Controls to detect background hydrolytic activity in the assay were undertaken for each set of assays. This consisted of a tissue homogenate which had been boiled to remove all JH hydrolytic activity. A mass homogenate consisting of tissue from pupae and adults was used as a control across

sets of assays. However, comparisons between individual assays were generally only made within a set of assays performed concurrently.

Protein concentrations were determined by the method of Bradford (1976) using the Biorad Protein Assay kit. The reaction was performed in a Titertek 96-well plate in a total volume of 200ml and the absorbance was measured at 550nm in a Titertek Multiskan plate reader (Flow Laboratories). Bovine serum albumen (Sigma) was used as a standard.

Enzyme activities are expressed as pmoles of JHIII hydrolysed per minute per mg of protein or alternately as the pmoles of JHIII hydrolysed per minute per individual.

2.2.3 Data Analysis

Significant differences between enzyme activities at time points making up developmental profiles in juvenile and adult stages were determined by performing an analysis of variance (ANOVA). All specific activity values were logarithmically transformed before analysis.

Most data from the analysis of tissue distributions of JHE and JHEH activities in adult stages were not normally distributed. Therefore, comparisons among tissues, sexes and enzymes were analysed using non-parametric statistical tests (Kruskall-Wallis for three or more groups, or Mann-Whitney U Test for two group comparison). Parametric t-tests were used when data was normally distributed.

Statistical significance between groups was assumed if the probability for random chance was less than 5%. All statistical tests were performed using the StatView package for Macintosh.

2.3 Results

2.3.1 Temporal Distribution

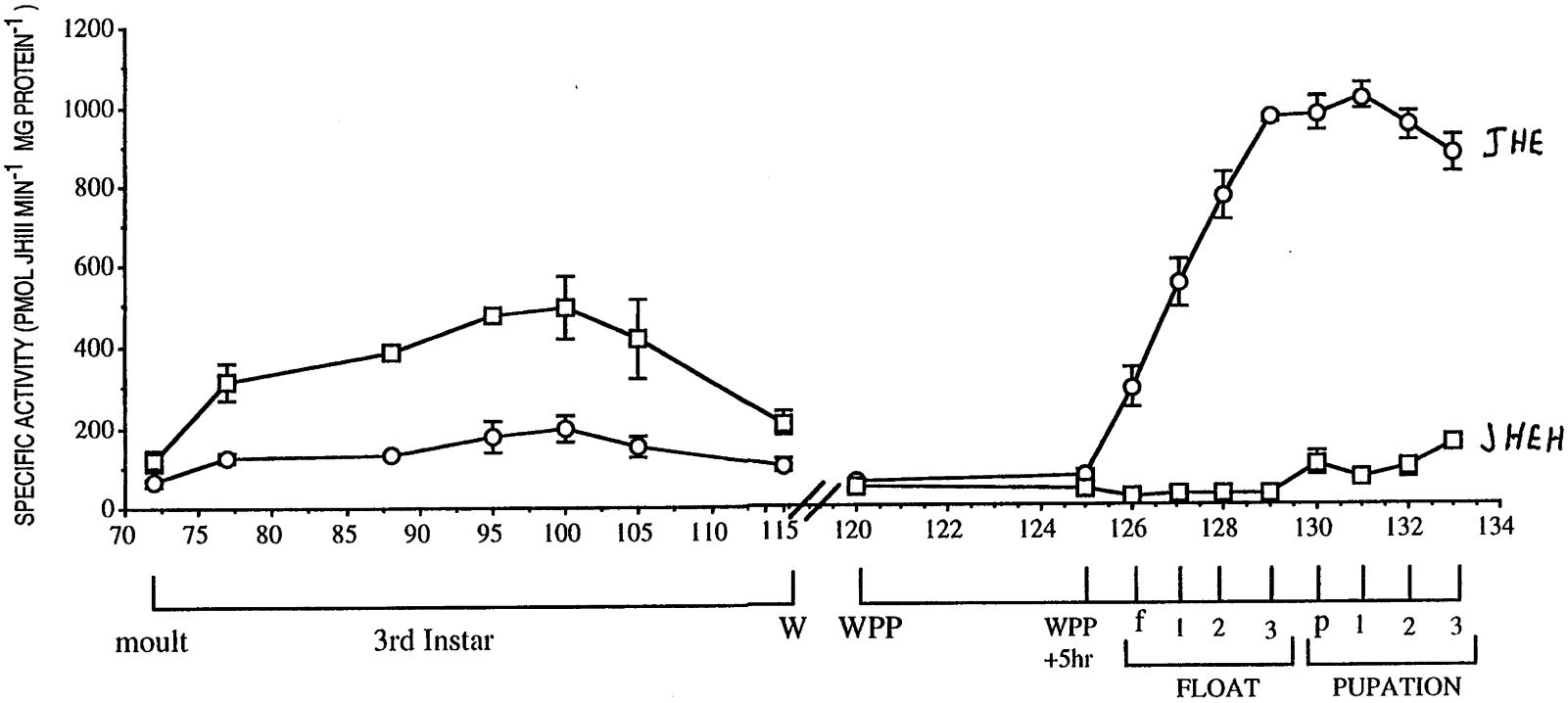
2.3.1.1. Larval/Pupal Developmental Profile

The detailed profile of JH hydrolysis during the last larval instar is shown in Figure 2.1. The analysis started at the moult to the third instar and hydrolysis was determined at intervals of 5 to 10 hours during the larval stage and hourly after pupariation until 3 hours after pupation was complete.

At the time of the moult to the third-instar both JHE and JHEH activities were very low. JHEH activity increased gradually during the prewandering stage with activity peaking at 88 to 105 hours. The increase in JHEH activity was not statistically significant among individual collection points except between the lowest JHEH activity measured at the moult and the highest JHEH activity measured at 100 hours. The peak in JHE activity mirrored the peak in JHEH activity but was not as pronounced. During the prewandering stage, JHEH activity accounted for approximately 73% of total JH hydrolysis. Finally, at the wandering stage, JHE and JHEH activities were at levels similar to those at the start of the third-instar.

In the prepupal stage, JHE activity clearly reached a peak at three hours after development to the floatation marker when the prepupae become bouyant (stage P3 of Bainbridge and Bownes, 1981). The specific activity of JHE rose approximately 14-fold within about four hours from immediately prior to development to the float marker and peaking three hours later just before head eversion and pupation (stage P4(ii)). JHE activity remained consistently high until at least three hours after pupation. During the prepupal stage JHE activity accounted for approximately 93% of JH hydrolysis (range across stages as above). The prepupal peak in JHEH activity was not absolutely defined because the activity appears to be still rising at the last developmental stage tested, however, some points can be made.

FIGURE 2.1 Developmental profile of JHE and JHEH activities in the final larval instar of *D. melanogaster* from the moult to the third instar until three hours after pupation. The scale for the larval stage is broader than for the prepupal and pupal stages because the larval stages were measured about every 5-10 hours while the prepupal and pupal stages were measured every hour starting five hours after pupation.
Circles indicate JHE activity and squares indicate JHEH activity. Each collection time point is the mean of 3-6 replicate assays. Each replicate for the larvae consisted of 30-50 larvae and 7-15 prepupae and pupae. In some cases the standard errors do not exceed the thickness of the line. 'W' refers to wandering, 'WPP' refers to pupariation, development to the float marker used by Mitchell and Mitchell (1964) began at 'f' and in this study continued for about three hours as indicated by the numbers after 'f', pupation occurred at 'p' and was monitored for a further three hours.



Firstly, although JHEH activity at the white prepupal stage was comparatively low, activity at the float marker stage was significantly lower and stayed at low levels until pupation occurred. Secondly, JHEH activity increased significantly at pupation and may have continued to increase for some hours after pupation.

2.3.1.2 Adult Developmental Profile

JH hydrolytic activity in virgin adults was determined from eclosion to two days post eclosion as shown in Figure 2.2.

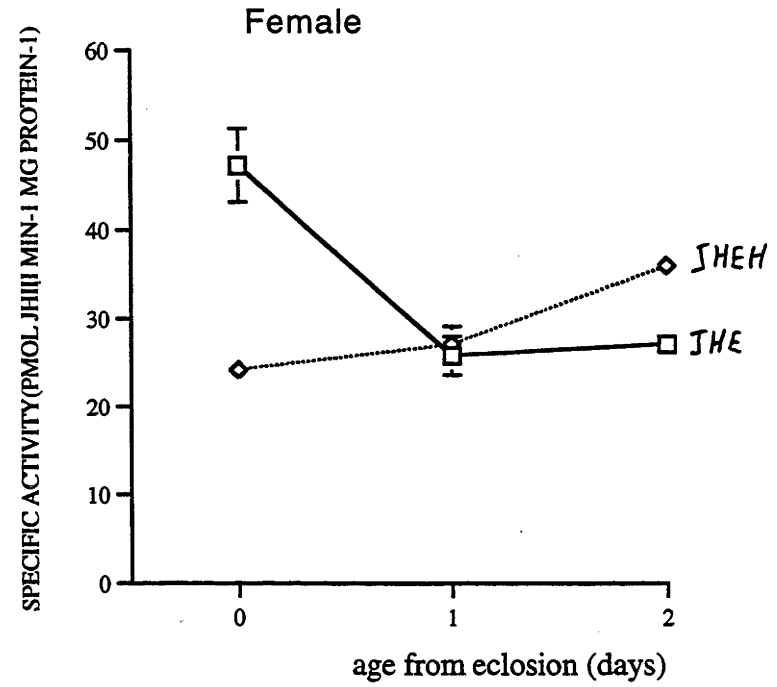
Newly eclosed male and female adults had the highest JHE activity over the two day period measured. By one day after eclosion the JHE activity had dropped by approximately 55% in females and approximately 42% in males and did not change significantly over the ensuing day. In contrast, JHEH activity in females remained at a constant level until two days post eclosion when it increased slightly but significantly ($p=0.05$). In males, the JHEH activity remained at a constant level over the two days monitored.

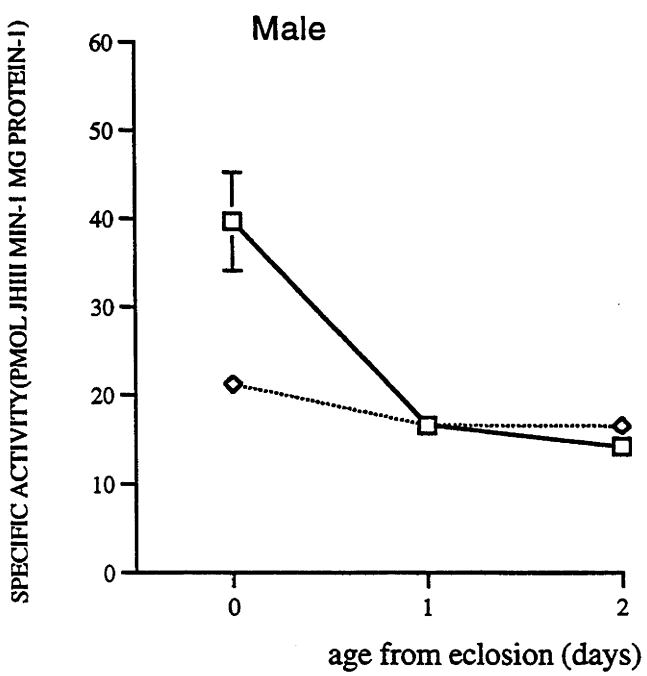
At eclosion, JHE activity was significantly higher than JHEH activity in both females and males but by one day after eclosion JHE and JHEH activities were at similar levels. By two days post eclosion JHEH activity was the major JH hydrolytic enzyme in females while in males, JHE and JHEH had similar levels of activity.

2.3.2 Tissue Distribution in Adults

To localise JH hydrolytic enzymes in adults a series of dissected body parts were assayed for JHE and JHEH activities. Firstly, the general distribution of JHE and JHEH activities was determined in heads, thoraces and abdomens (Figure 2.3). Secondly, JHE and JHEH activities were described for specific tissues or groups of tissues within the head, abdomen and thorax (Figures 2.4 and 2.5).

Figure 2.2 JHE and JHEH specific activities in virgin adult females and males at eclosion and one and two days later. Homogenates were pooled from 10-15 adults per replicate and the means of 3 replicates plotted. The squares represent JHE activity and the diamonds represent JHEH activity. In some cases the standard errors do not exceed the thickness of the line.





To allow direct comparisons of JH hydrolytic activities in segments and tissues, enzyme activities were calculated as activity (pmol JHIII per min) per individual.

2.3.2.1 Distribution in Segments

Females

In females, JHE is the predominant JH hydrolytic enzyme in the head, thorax and abdomen. JHE activity was highest in the abdomen with the head and thorax having similar levels of activity (73, 12 and 15% of total body JHE activity, respectively; Figure 2.3). JHEH activity was also detected primarily in the abdomen (about 97% of total body JHEH activity) with trace amounts in the head. No JHEH activity was detected in thoraces of females. JHEH activity was variable between replicates.

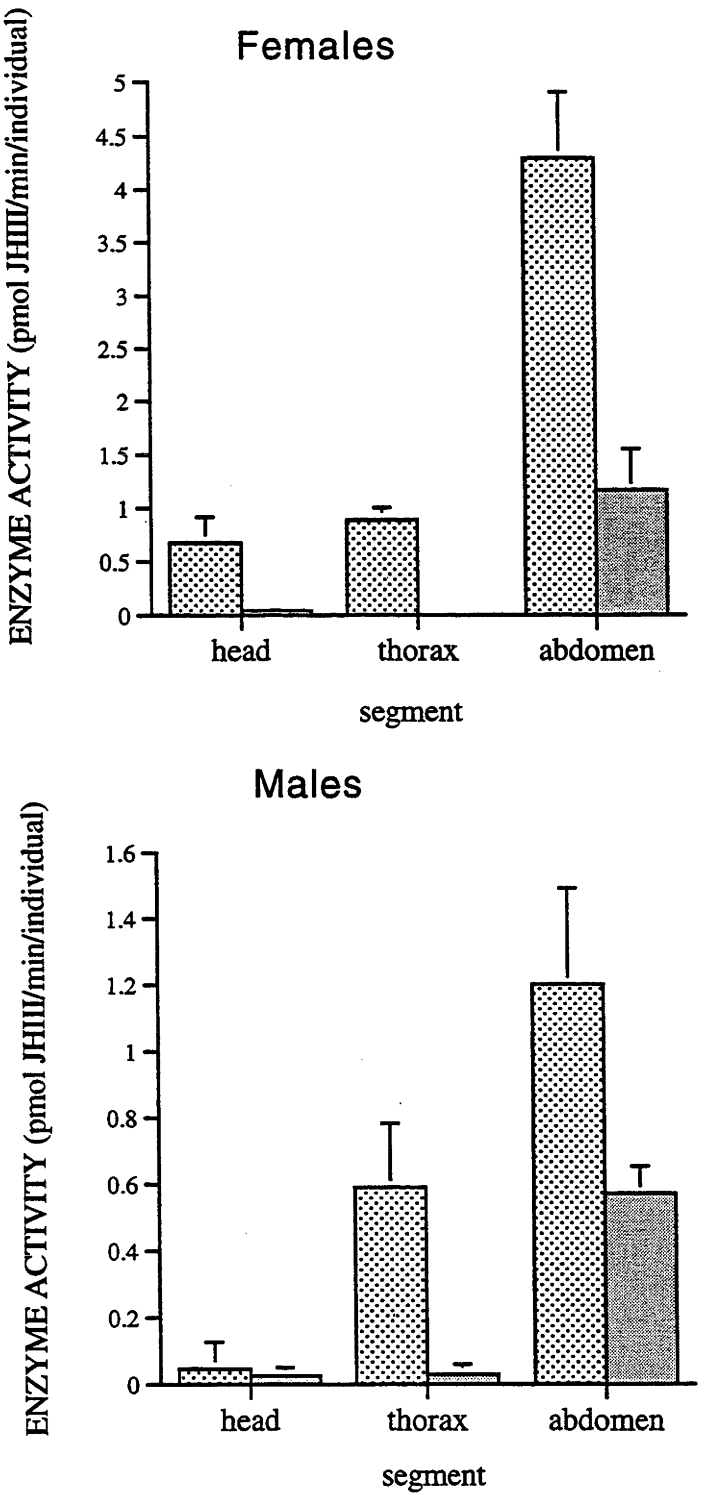
Males

In males, JHE is the predominant JH hydrolysing enzyme in the thorax and abdomen. JHE activity was at highest levels in the male abdomen (about 65% of total body JHE activity compared to 32% in thoraces and 3% in heads). JHEH activity was predominantly detected in the abdomens of males (about 91% of total body JHEH activity). There were only trace amounts of JHEH activity in the heads and thoraces of males.

Comparisons of JHE activity between males and females suggested that there were higher levels of JHE and JHEH activity in nearly all female segments. For example, in female abdomens JHE activity levels were about 3.6-fold higher than in male abdomens and JHEH activity levels were about two-fold higher. This may be attributable to the larger size of the females. However, as a proportion of total body activity male thoraces had higher activity levels than female thoraces, while female heads had higher activity levels than male heads.

JHE and JHEH Activities in Segments

Figure 2.3 JHE and JHEH activity per individual in female and male heads, thoraces and abdomens dissected from one day old virgin adults. Stippled bars represent JHE activity and solid bars represent JHEH activity. The mean activities of 3 replicate homogenates is illustrated together with the standard errors. Each replicate represents the activity from homogenates of 30 adults. In one case, the female thorax, no JHEH activity was detected and in some cases the standard errors do not exceed the thickness of the line.



In males and females, the abdomens contained the highest proportion of JHE hydrolytic activity.

2.3.2.2 Distribution in Tissues

Since all segments contained JHE or JHEH activity, tissues or tissue groups from all segments were considered (Figure 2.4). Tissues from the abdomen included the digestive system, the reproductive system and the carcass. The reproductive system was further dissected into the testes, accessory glands and duct/bulb for the males and the ovary and spermathecae/uterus for the females (Figure 2.5). Tissues from the head included the mouthparts, brains and carcass. Haemolymph was separated from the thorax.

Females

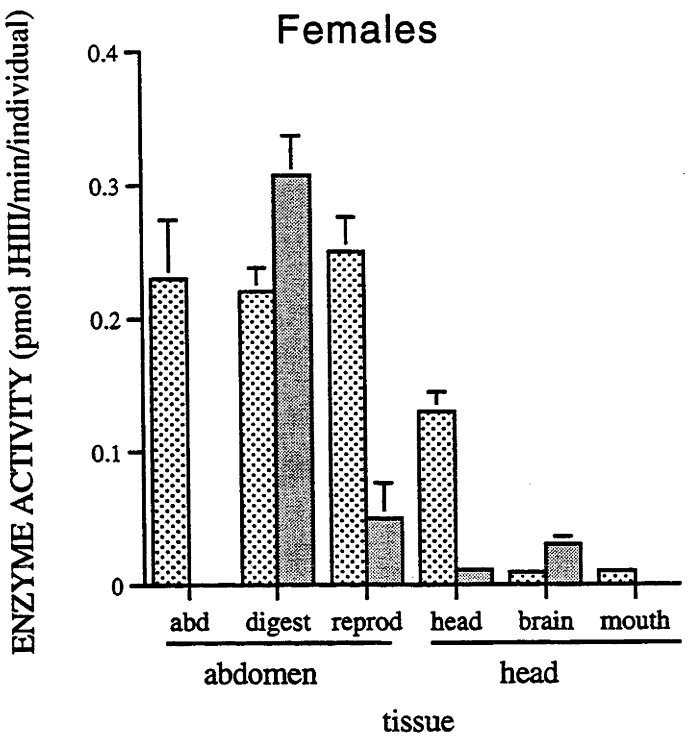
JHE activity was detected in all female tissues tested (Figure 2.4). In the head tissues of females, JH hydrolysis was predominantly due to JHE activity which was concentrated in the head carcass (about 87% of total head JHE activity). The mouthparts and brains of females had much lower levels of JHE activity (7 and 6% of total head JHE activity, respectively). JHEH activity was low in all head tissues and was undetectable in mouthparts.

In female abdominal tissues, JHE activity was distributed evenly between the carcass (abd), the digestive system (digest) and the reproductive system (reprod). JHEH activity was concentrated in the digestive tissues of females and at higher levels than JHE in this tissue (86% of total abdominal JHEH activity). Much lower levels of JHEH activity were detected in the reproductive system and in the carcass JHEH activity was barely detectable.

In female reproductive systems, JH hydrolysis was mainly due to JHE activity (Figure 2.5). JHE activity was concentrated in the ovaries with only a small amount of activity in the

JHE and JHEH Activities in Tissues

Figure 2.4 JHE and JHEH activity per individual in tissues dissected from one day old adults: abdominal carcasses (abd), digestive systems (dig), reproductive systems (rep), head carcasses (head), brains and mouthparts (mouth). Stippled bars represent JHE activity/individual and solid bars represent JHEH activity/individual. Each replicate represents 30 adults and the mean enzyme activities of three replicate homogenates are illustrated. In some cases no JHEH activity was detected and also in some cases the standard errors do not exceed the thickness of the line.



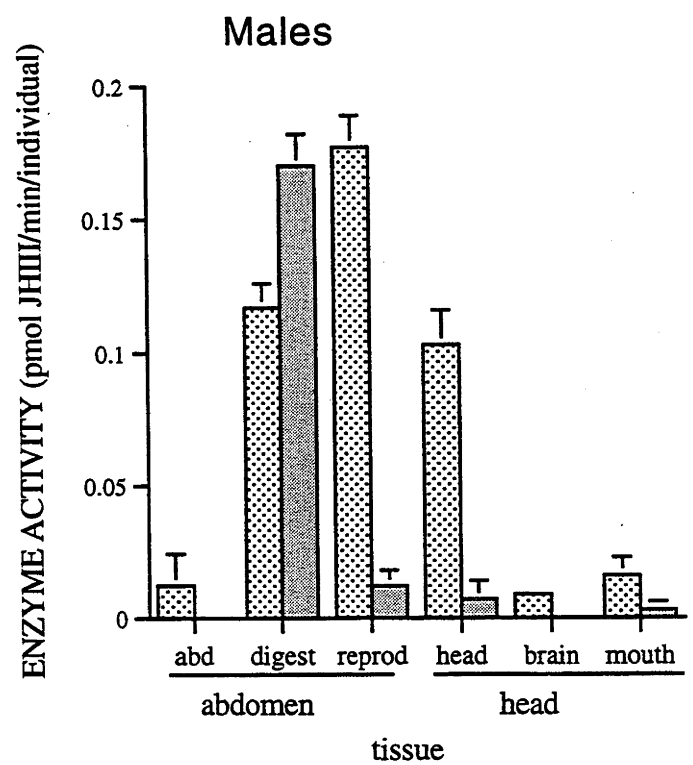
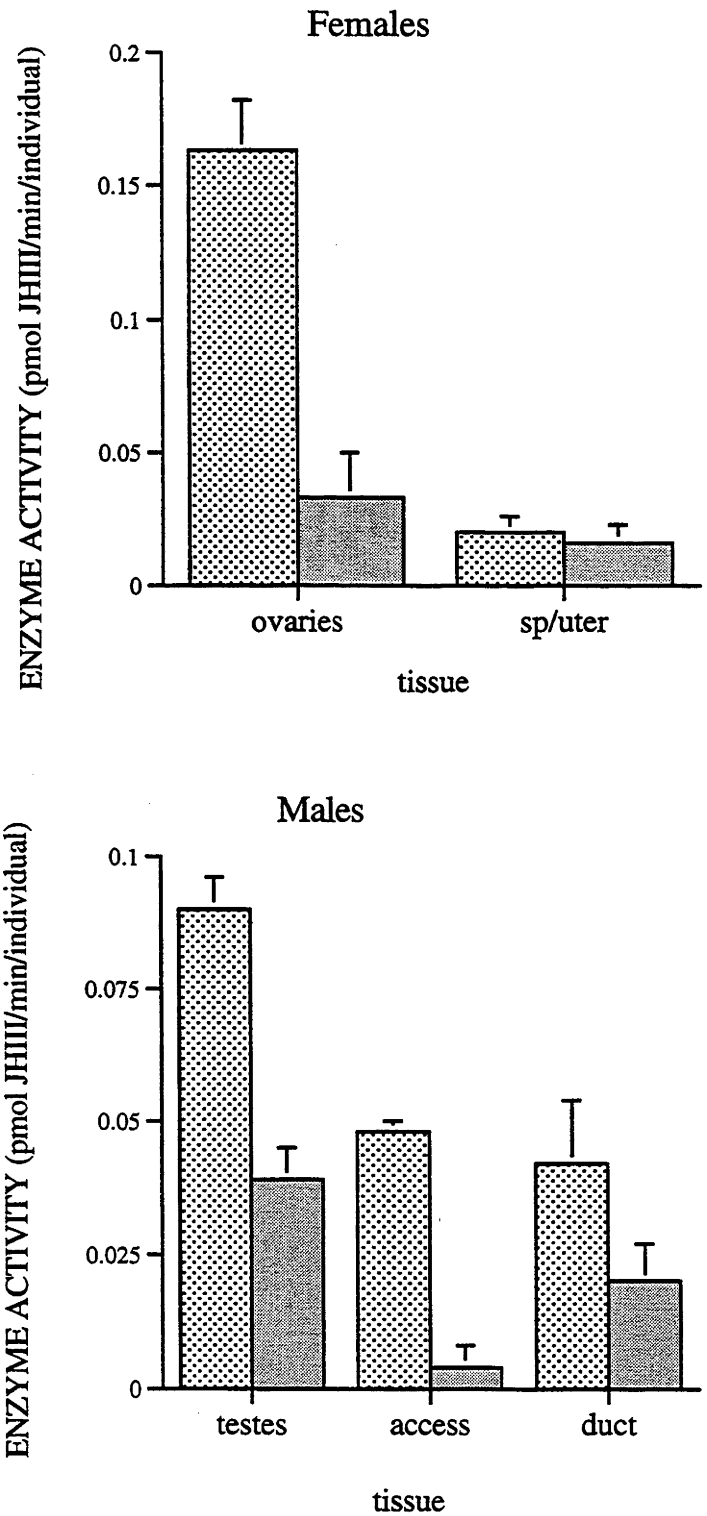


Figure 2.5 JHE and JHEH activity per individual in reproductive tissues of males; testes, accessory glands (access) and duct/bulb (duct) and females; ovaries and spermathecae/uterus (sp/uter). Stippled bars represent JHE activity/individual and solid bars represent JHEH activity/individual. Tissues were dissected from one day old virgin adults. Mean activities of three replicate homogenates are illustrated together with standard errors. Each replicate represents activity from homogenates of 30 adults.



spermathecae/uterus. JHEH activity was at low levels in the ovaries compared to JHE activity and at similar low levels to JHE activity in the spermathecae/uterus.

Haemolymph of females contained both JHE and JHEH activity. The specific activities of the two enzymes were similar but were relatively low compared to specific activities in the other tissues analysed (12.2 and 18.9 pmol JHIII/min/mg protein respectively; specific activities for other tissues are not shown).

Males

JHE activity was detected in all male tissues tested (Figure 2.4). In the head tissues of males, JHE activity was concentrated in the head carcass (about 80% of total head activity) and the mouthparts and brains of males had much lower levels of JHE activity (13 and 7% of total head JHE activity, respectively). JHEH activity was very low in all tissues with no activity detected in male brains.

In male abdomens, JHE activity was concentrated in the reproductive system with lower levels in the digestive system and only a trace amount in the carcass (58, 38 and 4% of total abdominal JHE activity, respectively). JHEH activity had a similar distribution in male abdomens as in females where it was concentrated in the digestive system and at a higher level than JHE activity. JHEH activity was also detected in the reproductive systems but at much lower levels. JHEH activity was at barely detectable levels in the abdominal carcass.

In male reproductive systems (Figure 2.5), JHE was the main source of JH hydrolysis. JHE activity was also concentrated in the testes with lower levels in accessory glands and duct/bulb (50, 27 and 23% of total reproductive system JHE activity, respectively). JHEH activity was concentrated in the testes with smaller amounts in the duct/bulb and accessory glands (62, 32 and 6% total reproductive system JHEH activity).

Haemolymph from males contained low levels of JHE and JHEH specific activities, compared to the other tissues analysed (16.7 and 8.9 pmol JHIII/min/mg protein respectively; specific activities for other tissues are not shown).

Comparisons between male and female tissues showed that JHE and JHEH activities had similar distributions in tissues in both sexes, with the exception of the abdomen carcass which had high JHE activity in females but not males.

2.4 Discussion

2.4.1 Temporal Expression of JH Hydrolytic Enzymes; Functional and Regulatory Significance

2.4.1.1 Juvenile stages

Generally, the profiles of JHE and JHEH activities measured in this study followed those described by Campbell *et al.*, (1992) with two definite peaks of JH hydrolysis at the prewandering and the prepupal stages. As documented previously for *D. melanogaster,* the changes in JH hydrolysis in the last larval instar are inversely related to the profiles of JH biosynthesis and JHIII titre in *D. melanogaster.* In addition, the JH hydrolysis and JH titre profiles in the last larval instar of *D. melanogaster* are similar to those observed in other insects (Campbell *et al.,* 1992). As such it is likely that JHE and JHEH have roles in *D. melanogaster,* similar to those in other insects at this stage of development i.e. regulation of development by scavenging the last traces of JH. Additional evidence for the role of JHE activity in metamorphosis is that inhibition of the peak of JHE activity in prepupae by treatment with a JHE inhibitor resulted in pupal death (Rauschenbach *et al.,* 1991).

At the moult to the last instar, JH hydrolytic activity in *D. melanogaster* was low which is similar to other insects (Roe and Venkatesh, 1990). At this stage of development JH titre and JH synthesis by the ring gland in *D. melanogaster* are unknown. However, in other insects, such as *L. cuprina* and *M. sexta,* JH biosynthesis is known to occur and/or the JH titre is high (Baker *et al.,* 1987; Sutherland and East, 1997). JH titre is believed to be high at this time in lepidopterans so that larval-specific structures are maintained and metamorphosis does not occur prematurely (Riddiford, 1994).

The prewandering and prepupal peaks of JH hydrolysis in *D. melanogaster* reported here and elsewhere are composed mainly of JHEH and JHE activity, respectively. While this composition is similar to what is observed in some lepidopterans (e.g. *T. ni*, Kallapur *et al.,* 1996) it contrasts with others such as *M. sexta*, where JHEH activity is the major component of both peaks (Jesudason *et al.,* 1992).

The data also suggest that the JH hydrolytic enzymes are temporally and differentially regulated. Firstly, the prewandering larval peak of JH hydrolysis was very broad and contrasts with the sharp peak of JH hydrolysis observed in most other insects, in particular lepidopterans, at this stage of development. This broad peak may have been an artifact of the experiment because larvae were aged from hatching rather than by developmental markers and it was evident that the individual larvae did not age synchronously. Many developmental changes occur in a relatively short time period at this stage in *D. melanogaster.* However, it is possible that the broad prewandering peak arises because of small differences in the timing of peak activities in individual tissues. In *T. ni* larvae, such variations in the time of JHEH peak activity have been observed (Kallapur *et al.,* 1996).

Secondly, the peak of prepupal JH hydrolysis in *D. melanogaster* was readily defined with JHE activity increasing sharply about six hours after pupariation and peaking about an hour prior to pupation. The changes in JHE and JHEH activities were not synchronous at the prepupal stage with JHE activity rising while JHEH activity was either decreasing or still

low. Differential regulation of JHE and JHEH activities has also been observed in *T. ni* larvae, while temporal regulation of these enzymes has been reported in all insects so far studied (Kallapur *et al.*, 1996; Roe and Venkatesh, 1990; de Kort and Granger, 1996).

2.4.1.2 Adult stages

The developmental profiles of JH hydrolytic enzymes in adults are consistent with other studies in *D. melanogaster* and suggest that JHE and JHEH activities are regulated temporally, differentially and sex-specifically (Yu and Terriere, 1978a; Campbell *et al.*, 1992). In *D. melanogaster* adults, there is no obvious correlation between JH hydrolytic enzymes and JH whole body titre and biosynthesis as found in juvenile stages, however, such correlations have been observed in other dipteran species such as *A. aegypti.* Female adults of this species require different levels of JH in a reproductive cycle; high levels of JH after emergence are necessary for development such as previtellogenic growth, and low levels of JH are necessary after a blood meal so ecdysteroids can play their role. The decreases in JH observed, are believed to be at least partly due to JH hydrolysis by JHE (Shapiro *et al.*, 1986; Borovsky *et al.*, 1992). By comparison, *Drosophila* undergo continuous egg production and maturation with no clearly defined cycle although different stages of reproduction require different levels of JH. Khlebodarova *et al.*, (1996) argued that regulation of JH titre in the reproductive tissues of adult female *D. melanogaster* is achieved by intense synthesis and rapid degradation of JH to provide the required levels of JH in different reproductive tissues.

The relationship between JH titre and biosynthesis and JH hydrolysis in *D. melanogaster* may be complex compared to other insects in part due to the fluctuating titres of different JH analogues. At eclosion a high JH titre is believed to be important for the initiation of vitellogenin synthesis and the development of mature gametes, ovaries and testes (Bownes and Rembold, 1987). Twenty-four hours after eclosion, JHIII titre and biosynthesis have declined but JHIII bisepoxide biosynthesis has increased and is temporally coincident with the increase in vitellogenesis and build up of yolk proteins, histolysis of larval fat body and

changes in sexual behaviour which all are believed to be affected by JH (Alteratz *et al.*, 1991; Riddiford and Truman, 1993). It is interesting then to note that the profile of JHE activity is similar to the JHIII profiles but inversely related to the JHIII bisepoxide profiles while JHEH activity changes little over the period investigated. The most likely explanation for high titres of JHE activity and JHIII at eclosion is that JHE is hydrolysing JHIII in particular tissues only. The changes in JHEH activity in whole females are more subtle than those detected for JHE activity and suggest that JHEH activity is hydrolysing JH at a constant low rate in single or multiple tissues.

2.4.2 Spatial Expression of JH Hydrolytic Enzymes; Functional and Regulatory Significance

This study suggests that JH hydrolytic enzymes are both abundant and ubiquitous in *D. melanogaster* adults and JHE and JHEH activities are regulated differentially, showing both tissue and sex specificity. JHE activity was found in all male and female tissues tested and was generally more abundant than JHEH activity, the exception being the digestive system.

2.4.2.1 Females

High levels of JHE activity in particular, were detected in female and male reproductive systems and this is consistent with the results of a number of studies demonstrating a role for JH in reproductive tissues. In *D. melanogaster* females, JH is believed to stimulate ecdysteroid production by the follicle cells in the ovary and to regulate the synthesis of yolk proteins and induce their uptake into the oocyte (Gavin and Williamson, 1976; Jowett and Postlethwait, 1980; Koeppe *et al.*, 1985; Schwartz *et al.*, 1985; 1989; Bownes, 1989). In other species, JH may influence the growth of follicles and follicular epithelium (Gwadz and Spielman, 1973; Shapiro and Hagedorn, 1982; Wyatt and Davey, 1996). In some insects, JH has also been implicated in certain processes in the spermathecae (Wyatt and Davey, 1996). In *D. melanogaster* females, JHE and JHEH activity were detected in the ovaries and

spermathacea/uterus suggesting that JH hydrolysis may regulate the titre of JH in these tissues in one-day old virgin females.

Another tissue in females containing high levels of JHE activity was the abdominal carcass. The abdominal carcass is the major site of fat body in females who have large stores of this tissue for reproduction. In *D. melanogaster,* JH is believed to prime the fat body to respond to 20-hydroxyecdysone which then stimulates synthesis of yolk proteins in the fat body (Schwartz *et al.,* 1985; 1989; Bownes, 1989). Several lines of evidence suggest that the high concentration of JHE activity observed in the female abdominal carcass is most likely associated with fat body. In the mosquito, *A. aegypti*, adult fat body and ovaries produce similar JHE activities in organ culture. The fat body of third-instar larvae of *D. hydei* also were shown to have high levels of JHE activity (Klages and Emmerich, 1979; Shapiro *et al.,* 1986). In *T. ni* and *M. sexta* larvae, fat body has been implicated as the major site of JHE synthesis and JHE is regulated by JH in this tissue (Wing *et al.*, 1981; Jones and Hammock, 1983; Jesudason *et al.,* 1992). In *D. melanogaster* females, all the JH hydrolytic activity in the abdominal carcass was accounted for by JHE with no JHEH activity being detected. This suggests that the fat body may be a major site for JHE production and/or in this tissue JHE activity is solely responsible for hydrolysis of JH.

2.4.2.2 Males

JH hydrolytic enzymes were also found to be concentrated in the reproductive tissues of males which included the testes, which had the highest concentration, the accessory glands and ejaculatory duct/bulb. JH has been implicated in many reproductive processes in male insects and treatment of these tissues with JH causes various effects. One of the most notable effects of JH on the male reproductive system is in stimulating growth of accessory glands. In organ culture, JH in the presence of calcium stimulated a nearly three-fold increase in protein synthesis in the accessory glands of *D. melanogaster* males (Yamamoto *et al.,* 1988). In other insects, including dipterans, JH has also been implicated in growth and secretions

from the accessory glands (Ogiso and Takahashi, 1984; Fernandez and Klowden, 1995; Wyatt and Davey, 1996). In the testes of some insects, JH has been found to affect the level of ecdysteroids (Wyatt and Davey, 1996). These observations suggest that in *D. melanogaster*, regulation of JH titre in reproductive tissues is at least partly controlled by degradation.

2.4.2.3 Both sexes

Relatively high levels of both JHE and JHEH activity were detected in the digestive systems of females and males which is consistent with observations from other Diptera and Lepidoptera (Jesudason *et al.*, 1992; Grieneisen *et al.*, 1995; Kallapur *et al.*, 1996). Interestingly, in *D. melanogaster*, the digestive system was the only tissue where similar levels of JHE and JHEH activity were detected. The same observation has also been recorded for blood-fed female *C. quinquefasciatus* (Lassiter *et al.*, 1996).

The role of relatively high levels of JH hydrolytic enzymes in digestive tissues is unknown. One possibility is that after eclosion, lepidopterans swallow the moulting fluid which is likely to also contain JH (Reynolds and Samuels, 1996). High levels of JH hydrolytic enzymes in the gut would clear the body of JH which may interfere with other processes at this life stage. The digestive tissues include the Malphighian tubules which are specialised for processing and removal of compounds from the insect circulatory system and the midgut which is a site of digestive enzyme production, digestion and absorption (Jesudason *et al.*, 1992). It is possible that other enzymes, in the midgut especially, are also metabolising JH. It has recently been recorded that a cytochrome P450 terpenoid hydroxylase, (CYP4C7), found predominantly in the corpora allata of cockroaches is also detected in trace amounts in the midgut (Sutherland *et al.*, submitted for publication). This enzyme metabolises JHIII and other JH-like compounds (Sutherland *et al.*, submitted for publication). Such a finding suggests that it is possible that there are enzymes in the midgut of *D. melanogaster*, other than JHE and JHEH that are able to metabolise JHIII. The radiometric partition assay used

in this study assumes that the enzymes hydrolysing JHIII in adults are JHE and JHEH (Campbell *et al.*, 1992). However, the assay was not characterised at the stage of development used to study enzyme distribution in tissues and it is also possible that the products of JH hydrolysis characterised by thin layer chromatography, JH acid and JH diol, were produced by enzymes other than JHE and JHEH.

The only tissue in the heads of males and females that had a substantial level of JH hydrolytic activity was the carcass, which had predominantly JHE activity. The head carcass includes the antennae, epidermis, cuticle and very small amounts of fat body and the role of JH in these tissues is not known. It is interesting to note that a larval serum protein, LSP2, in *D. melanogaster* is also found at its highest levels in the fat body of heads. Although the function of LSP2 is unknown, it is induced by 20-hydroxyecdysone. Another larval serum protein in *D. melanogaster*, LSP1, is essential for fertility of the female (Roberts, 1987)

Chapter Three

Regulation of JH Hydrolytic Enzymes by JH

3.1 Introduction

Studies on various insects have demonstrated the existence of a regulatory feedback mechanism whereby an increase in JH titre stimulates an increase in JHE activity which in turn regulates JH titre. Previous studies in several Lepidoptera have demonstrated that JH plays a crucial role in regulating JHE activity both indirectly in last instar prewandering larvae and directly in prepupae (Roe and Venkatesh, 1990; Wroblewski *et al.* 1990; Venkataraman *et al.* 1994). JH has not been shown to regulate JHE activity in any adult Lepidoptera yet examined. The regulation of JHEH activity in larvae, pupae or adults has not been as widely studied as that of JHE activity, however, in studies of Lepidoptera, JHEH activity was not regulated by JH (Jesudason *et al.* 1992).

Methoprene was one of the first juvenoids tested as a insect growth regulator (Staal, 1986). Methoprene and epofenanone have very high activity in Diptera and Lepidoptera and are powerful stimulants of JHE activity in lepidopteran larvae (Sparks and Hammock, 1982; Venkatesh and Roe, 1988). Little information is available on JH or JH mimics regulating the activity of JH hydrolysing enzymes in dipterans. However, in *C. quinquefasciatus*, JHE activity was not affected by treatment with methoprene in larvae or pupae but was affected in female adults. JHEH activity in *C. quinquefasciatus* larvae was repressed in response to treatment with methoprene but methoprene did not affect JHEH activity at any other stage (Lassiter *et al.* 1995; 1996).

In the study of *D. melanogaster* described in this chapter, regulation of JHE and JHEH activities by JH and JH mimics was examined in last-instar larvae, prepupae and adults. The study identified affects in whole adults that were further examined in individual tissues. It

was predicted that JH and JH mimics would have their greatest affects on hydrolytic activity in reproductive tissues of both females and males. This prediction was based on: observations from similar studies on other insects, the information described in chapter 2 demonstrating that high levels of JHE and JHEH are present in these tissues and the evidence that JH plays a physiological role in these tissues.

3.2 Materials and Methods

3.2.1 Strains and Culturing Conditions

The *D. melanogaster* strain, 12Ill.2 (Cooke *et al.* 1987) was used throughout this study. All organisms were cultured and aged as described in Chapter 2.

3.2.2 JHE/JHEH Assays

Tissues were stored, homogenised and the enzymes assayed as described in section 2.2.2. Enzyme activities are expressed as pmoles of JHIII hydrolysed per minute per mg of protein or as the pmoles of JHIII hydrolysed per minute per individual.

3.2.3 Treatment Procedures

JHIII (Sigma/Fluka) and epofenanone (a kind donation from Stephen Trowell) were diluted in ethanol and S-methoprene (Zoecon) was diluted in acetone or ethanol and 0.2ml was topically applied to the ventral surface of each larvae or pupae. For adults the compounds were applied to the dorsal surface of the abdomen. All experiments included organisms treated with the solvent only and where possible untreated organisms were included to test for effects of the solvent. Experiments on whole adults and larvae were performed in triplicate, the segment dissections were undertaken six times and the tissue dissections were

undertaken five times. For each replicate of whole adults, 6-10 individuals were pooled prior to assay. For each replicate of larvae or dissected adults, 30 individuals or tissues from 30 individuals were pooled for each assay.

3.2.3.1 Larvae

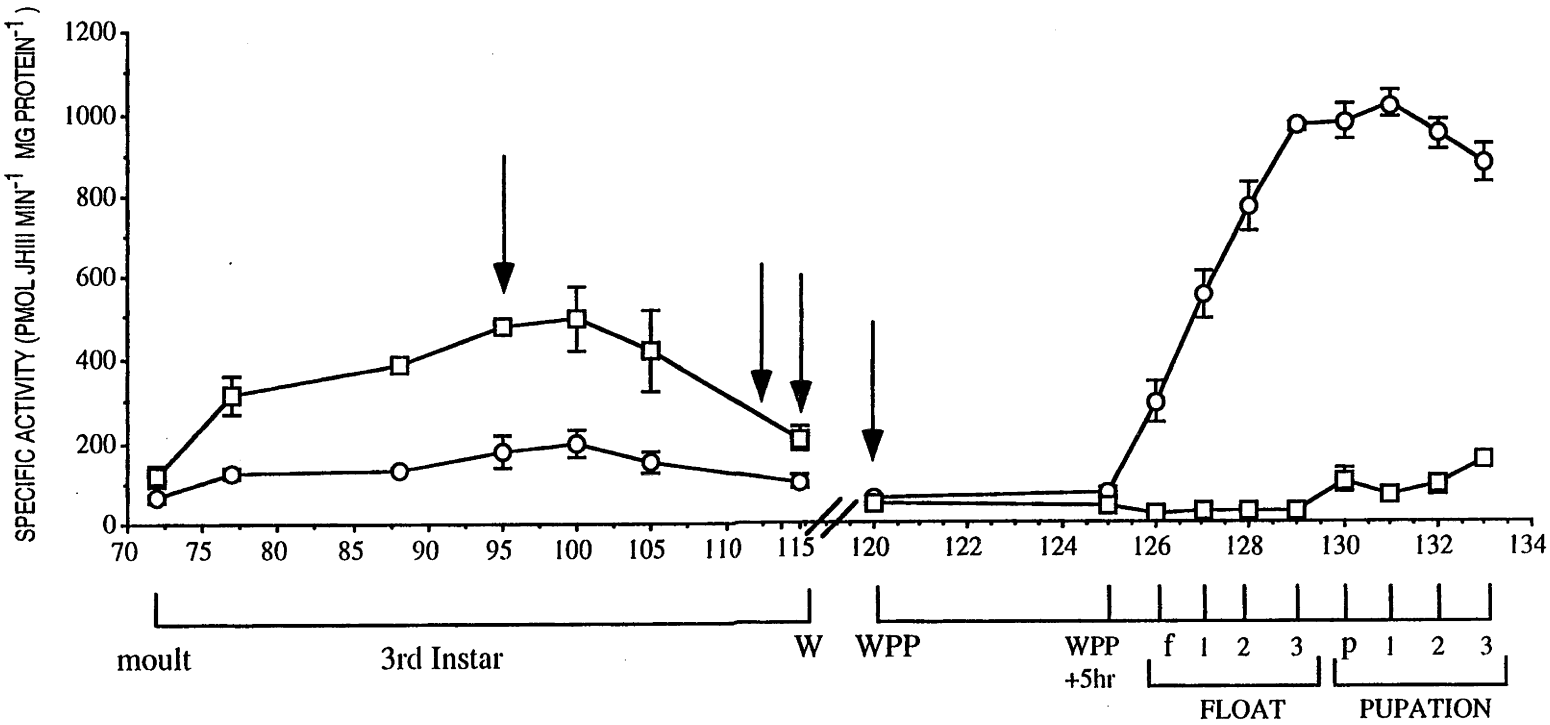
The effect of the JH mimic, methoprene, on JHE and JHEH activities in larvae was examined after treating four larval stages; feeding, early and late wandering larvae and white prepupae (WPP). The effect of methoprene on JHE and JHEH activities in 95 hour feeding larvae (i.e. larvae aged 95 hours from hatching) was determined by topically applying 5µg methoprene. Methoprene 5 or 20µg, was also topically applied to early (112 h) and late (118 h) wandering larvae and WPP (Figure 3.1). JH hydrolytic enzyme activities were assayed at the various times as described in Table 3.1.

Table 3.1. Various life stages were treated with methoprene then assayed for JHE and JHEH activities at different times after treatment, which corresponded to particular life stages assayed.

	Time assayed	
Life stage treated	(hours after treatment)	Life stage assayed
feeding larvae	8	feeding
early wandering	6-6.5, 8	wandering or prepupae
late wandering	4,6,7,8,10,24	prepupae
WPP	3,4,5,6,8,18,19	prepupae/pupae

Timing of Methoprene Treatments in Larvae/WPP

FIGURE 3.1 Developmental profile of JHE and JHEH activities in the final larval instar of *D. melanogaster* from the moult to the third instar until three hours after pupation. See Figure 2.1 for explanation of figure.
Arrows indicate the approximate times of methoprene treatment of larvae. Feeding larvae (about 95 hours) were topically treated with 5µg methoprene and assessed 6-8 hours after treatment. Early and late wandering larvae and white prepupae were topically treated with 5 or 20µg methoprene and assessed at various times after treatment (see Table 3.1 for times).



3.2.3.2 Adults

The effect of JH or JH mimics on JHE and JHEH activities in adults was examined by topically treating newly eclosed adults with various compounds and then determining enzyme activities one day after treatment. Activities from treated adults were compared to solvent treated and untreated adults of the same age. Initially, whole adults were treated with either 1, 2.5 or 5µg JH, 5µg methoprene or 1µg epofenanone and enzyme activities assessed one, or in some cases four days, after treatment. Secondly, adults were treated with 2.5µg JH or 2.5 or 5µg methoprene, dissected one day after treatment into body segments; heads, thoraces and abdomens and enzyme activities determined in the segment. In the final study adults were treated with 5µg methoprene, dissected into separate tissues one day after treatment and enzyme activities determined in the dissected tissues. The head and abdomen were dissected as described in Chapter 2 with the exception that the reproductive system was not dissected into individual tissues or groups of tissues. Haemolymph was extracted from the thoraces.

3.2.4 Statistical Analysis

The data were not normally distributed so various non-parametric tests were used to analyse the data depending on how the data were compared. The effects of methoprene on different larval and prepupal stages were analysed by the Mann-Whitney U test. The effects of JH and JH mimics on enzyme activities in whole adults were analysed using paired t-tests or Kruskal-Wallis tests depending on the nature of the data. A paired t-test was used for data where the variables were paired and a non-parametric test was not as sensitive. Enzyme activities in dissected segments were analysed using the Mann-Whitney U test and activities in dissected tissues were analysed using Wilcoxen Signed Ranks paired test. The statistical tests were performed using the computer package StatView (Abacus Concepts).

3.3 Results

3.3.1 Effects of Methoprene on JH Hydrolytic Activities in Larvae and Pupae

Any effects on JHE and JHEH activities that could be attributed to the solvents, acetone and ethanol, were assessed by comparing the activities in untreated and solvent treated organisms of similar ages. Topical application of the carrier solvent, acetone, did not affect JHEH activity in any of the treatment times tested (data not shown). However, acetone did have a small but significant effect on JHE activity at one time point following treatment; activity was reduced by about 1.7-fold in larvae treated in the early wandering stage and assayed 8 hours after treatment ($Z = -1.964$, $p<0.05$; data not shown).

Topical application of methoprene to feeding or wandering larvae did not influence the activities of JHE or JHEH at any time up to 24 hours after treatment (Table 3.2). However, methoprene treatment of WPP did produce small but significant affects on JHE activity at two of the time points analysed. Three hours after treatment with 5µg methoprene JHE activity was repressed by 1.2-fold and nineteen hours after treatment with 20µg methoprene JHE activity was increased 1.3-fold. JHEH activity was not affected in WPP treated with methoprene.

3.3.2 Effect of JHIII and JH Mimics on Adult JH Hydrolytic Activities

3.3.2.1 Whole Adults

To determine if JH or JH mimics modulated JHE or JHEH activities, newly eclosed adults were treated with JHIII or the JH mimics, epofenonane and methoprene, and assayed one day after treatment. In addition, adults treated with JHIII were assayed four days after treatment. Neither of the carrier solvents, acetone nor ethanol had any effect on JH hydrolysing enzyme

Table 3.2 Z values calculated using Mann-Whitney U test for differences in JHE and JHEH activities of organisms treated with methoprene compared to solvent treated controls. Values are given for four stages of treated larvae; feeding, early and late wandering larvae and white prepupae. Assay times for JHE and JHEH activities are written in hours (hr) after treatment and stage collected as wandering (wand) or prepupae (PP).
nd=no activity detected in either solvent controls or treated organisms.
*= significant at $p \leq 0.05$

Treatment	JHE Z value	JHEH Z value
Early wandering larvae		
6-6.5hr wand	-0.66	-0.66
6-6.5hr PP	-0.22	-0.47
8hr wand	-0.66	-0.66
8hr PP	-0.22	-0.89
10hr PP	-1.09	-0.66
Late wandering larvae		
4hr PP	-.1.09	-0.22
6hr PP	0	-0.23
8hr PP	-0.11	-1.16
10hr PP	-1.09	-0.70
7hr (20mg)	-0.73	nd
24hr (20mg)	-0.22	0
WPP		
3hr PP	-1.96*	-0.22
4hr PP	-0.22	nd
5hr PP	-0.22	nd
8hr PP	-0.66	nd
10hr PP	-0.22	nd
18hr PP	-0.22	nd
6hr (20mg)	-0.66	nd
19hr (20mg)	-1.96*	-0.66
Feeding larvae		
age	-0.22	0

activity (data not shown). No effect on JHEH activity was detected in any treatment on males or females (data not shown).

In males treated with 1µg epofenonane, JHE activity increased about 1.8-fold but there was no effect on JHE activity in females (Table 3.3, Figure 3.2a). However, in pilot experiments using higher concentrations of epofenonane (5 and 10µg), JHE activity increased in both males and females (data not shown).

Treatment with 5µg methoprene increased JHE activity in both males and females (Figure 3.2b). The increased activity observed in males was statistically significant with the tests used, however, the increase in activity in females was not (Table 3.3). This was due to the variability of the methoprene-induced increases of activity in individual assays. In males, the increase in activity in response to methoprene treatment ranged from 1.2- to 2.3-fold and in females the increase ranged from 1.1- to 2.4-fold.

The effects of three different amounts of JHIII were tested in adults; 1, 2.5 and 5µg. Effects of applications of JHIII on JHE and JHEH activities were assessed one and four days after treatment. For both males and females, JHIII increased JHE activity but the effects of three concentrations of JHIII on JHE activity were the same (all caused an increase in activity) and for both sexes the effects at one and four days old were also the same (Table 3.3). Therefore, data collected on JHE activity for the three amounts of JHIII at one and four days old were pooled for subsequent analysis. These data were pooled separately for males and females and plotted on a graph (Figure 3.2c).

The increases in JHE activity one day after treatment with JHIII were greater in males than in females. By four days after treatment the activity levels had decreased slightly in males and were similar to those found in females. JHE activity in males, increased in response to JHIII by approximately 1.4- to 1.6-fold. In females, JHE activity was increased approximately 1.3-fold in response to JHIII.

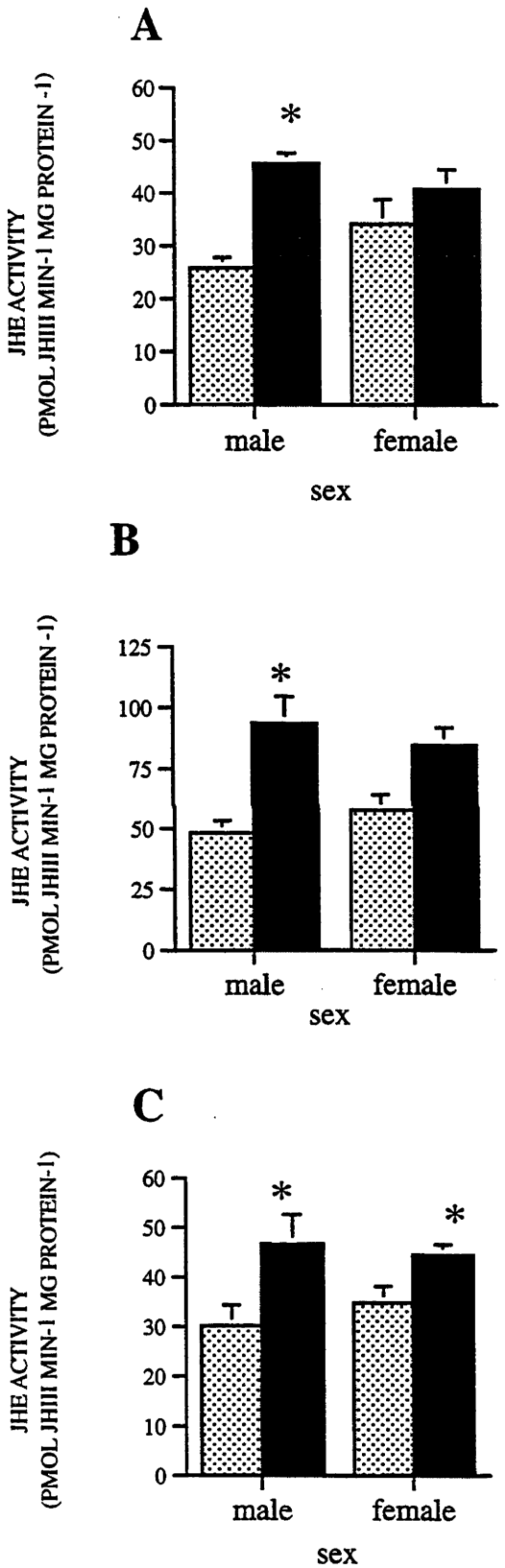
Table 3.3 Paired t and H values calculated using paired t tests or Kruskal-Wallis tests respectively. Data are from whole adult experiments treated with either JH or a JH mimic and JHE activity assayed one day after treatment. For JHIII, comparisons of doses used (1, 2.5 and 5μg), ages collected (1 and 4 days after treatment) and sex are included.

1 and 4do= one and four days old at collection

* = significant at $p \leq 0.05$

Treatment	Paired t value	H value
epofenonane		
female	-1.538	
male	-6.547*	
methoprene		
female	-2.113	
male	-2.985*	
JHIII		
female	-20.736*	
male	-8.671*	
dose effect		
female 1do		1.689
male 1do		3.289
female 4do		0.644
male 4do		3.954
age effect		
female	-0.413	
male	-0.589	
sex effect	-2.634*	

Figure 3.2 JHE activity per mg of protein in whole body homogenates of one day old adults treated with A) 1mg epofenonane B) 5mg methoprene or C) 1, 2.5 or 5mg JHIII combined. Homogenates from 6-10 adults represent one replicate and the means of three replicates are plotted. Stippled bars represent solvent-treated adults and solid bars represent JHIII- or JH-mimic-treated adults.
* indicates significance at $p \leq 0.05$



In summary, treatment of males with JHIII or the JH mimics methoprene or epofenonane increased JHE activity to a similar extent. By contrast, in females, epofenonane (at the concentration tested) had no effect on JHE activity, while both methoprene and JHIII increased activity, with methoprene having the greatest effect. Therefore, both JHIII and methoprene were used in further studies to identify the tissue specific locations where JHE activities may be modulated by JH and JH mimics.

3.3.2.2 Body Segments

To broadly localise the effects of JH and JH mimics on JH hydrolytic enzymes, enzyme activities in adult body segments were analysed after topical application of methoprene or JHIII.

Comparison of JHEH activities in body segments dissected from untreated and solvent-treated adults revealed that the solvents did not affect activity in any segment (data not shown). Similar comparisons of JHE activities revealed that both ethanol and acetone decreased JHE activity approximately 0.7-fold in both male and female heads while in male heads only, acetone decreased activities to a similar extent. Because of these solvent effects, activities in adults treated with JH or JH mimics were compared to activities in adults treated with solvent.

Initially, two amounts of methoprene were tested to determine the amount required to cause an effect on JH hydrolytic activity in adult body segments, heads, thoraces and abdomens. Treatment of adults with 2.5µg or 5µg methoprene did not affect JHEH activity in any body segment in females or males (data not shown). However, both amounts of methoprene stimulated JHE activity in all male segments and the head and thoracic segments of females. The JHE activity in the abdominal segments of females was not stimulated with either treatment. Since the data using a treatment of 5µg methoprene were more complete than

those using 2.5µg, the analysis of methoprene effects and comparisons between JHIII and methoprene discussed below, utilised the data from treatments with 5µg methoprene.

Methoprene treatment of adults significantly increased JHE activities in all body segments of males and the heads and thoraces of females (Table 3.4A, Figures 3.3 and 3.4). Comparisons of increases in JHE activity between segments suggested that in males the greatest increases were in the thoraces, although statistically there is little difference between the head and thoraces (1.5-fold and 1.7-fold, respectively; Table 3.4B). In females, mean fold increases in JHE activity were the same in heads and thoraces (both 1.7-fold). JHE activity in abdomens of methoprene treated females was also marginally higher (1.2-fold) than in solvent-treated individuals but the data were variable and so the effect was not statistically significant at the 95% confidence level.

The effects of JHIII treatment on JHE and JHEH activities in body segments were similar to those of methoprene. JHIII treatment of adults did not affect JHEH activity in any segment, except the male thoraces in which activity was increased (data not shown). However, JHEH activity in thoraces was too low to accurately estimate the magnitude of the effect. JHIII treatment increased JHE activity in all body segments of males and heads and thoraces of females (Table 3.4; Figures 3.3 and 3.4). JHE activities increased approximately 1.9- to 2-fold in all body segments of males and females that were responsive to JHIII. There were no effects in female abdomens.

Comparisons of the effect of methoprene and JHIII on JHE activities revealed that, in general, both compounds had similar effects on enzyme activities in most segments. The one exception was the male abdomen in which JHIII treatment le d to a greater increase in JHE activity than in response to methoprene treatment (Table 3.4C). Furthermore, there were no sex specific differences in the increases in JHE activity in response to topical application of JHIII or methoprene (Table 3.4D).

Table 3.4 Z values calculated using the Mann-Whitney U test on treated flies that were separated into segments. (A) Adults topically treated with methoprene or JHIII were dissected one day after treatment. JHE activities in hormone-treated adults were compared with solvent-treated adults. (B) Comparison between segments treated with methoprene or JHIII. JHE activity in hormone-treated adults was compared to activity in solvent-treated adults and expressed as a ratio. (C) Comparison of sex effects in hormone-treated adults. Female abdomens were not included in some analyses because no induction in JHE activity was detected. Comparisons were as for part (B). (D) Comparison of treatments with methoprene and JHIII in males and females. Comparisons were as for part (B).
*= significant at $p \leq 0.05$ ni = not included

A)

Treatment	**Z value**
methoprene	
female	
head	-2.406*
thorax	-2.892*
abdomen	-1.283
male	
head	-2.242*
thorax	-2.887*
abdomen	-2.887*
JHIII	
female	
head	-2.887*
thorax	-2.012*
abdomen	-0.802
male	
head	-2.887*
thorax	-2.887*
abdomen	-2.892*

B) segment comparison

Treatment	**Z value**
methoprene	
female	
head vs thorax	-0.48
head vs abd.	-1.761
thorax vs abd.	-2.882*
male	
head vs thorax	-1.601
head vs abd.	-0.48
thorax vs abd.	-2.242*

Table 3.4 cont.

JHIII

<u>female</u>	
head vs thorax	-0.401
head vs abd.	ni
thorax bs abd.	ni
<u>male</u>	
head vs thorax	-0.16
head vs abd.	-0.32
thorax vs abd.	-0.48

C) treatment comparison

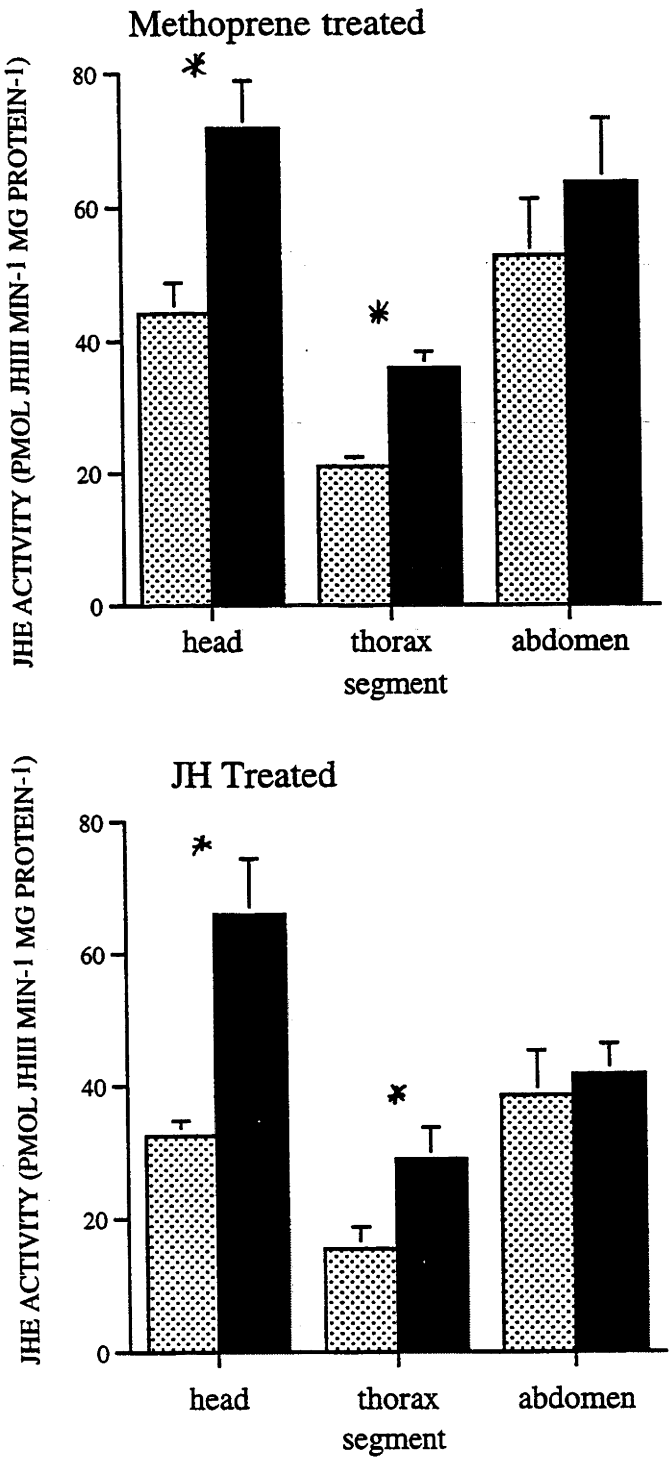
<u>female</u>	
head	-1.121
thorax	-0.964
<u>male</u>	
head	-1.922
thorax	-1.121
abdomen	-2.882*

D) sex comparison

<u>methoprene</u>	
head	-0.48
thorax	-0.16
abdomen	-1.441
<u>JHIII</u>	
head	-0.48
thorax	-0.16

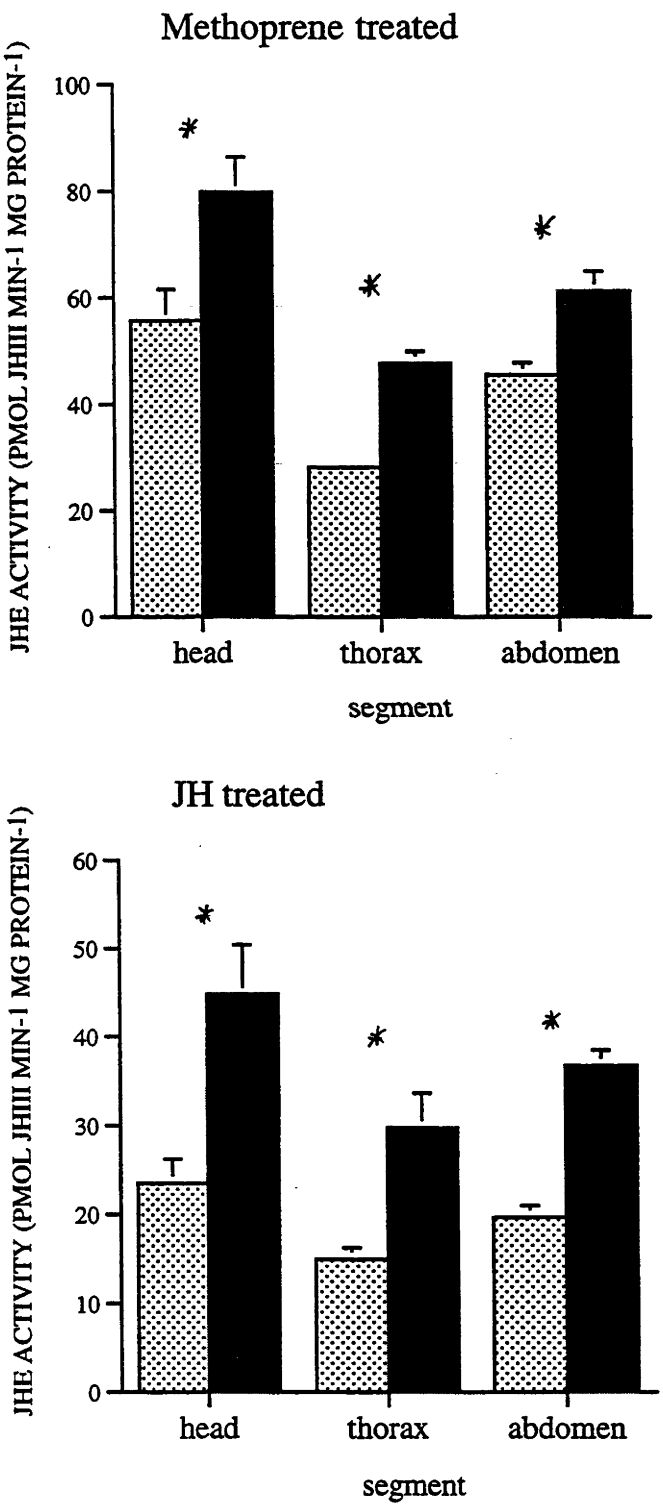
Female Segments Treated with Methoprene and JHIII

Figure 3.3 JHE activity per mg of protein in heads, thoraces and abdomens from females treated with 5mg methoprene or 2.5mg JHIII and assayed one day after treatment. Homogenates of segments from 30 adults represent one replicate and the means of 6 replicates are plotted. Stippled bars represent solvent-treated adults and solid bars represent JH- or JH-mimic-treated adults.
* indicates significance at $p \leq 0.05$



Male Segments Treated with Methoprene and JHIII

Figure 3.4 JHE activity per mg of protein in heads, thoraces and abdomens from males treated with 5mg methoprene or 2.5mg JHIII and assayed one day after treatment. Homogenates from segments of 30 adults represent one replicate and the means of 6 replicates are plotted. Stippled bars represent solvent-treated segments and solid bars represent JH- or JH-mimic-treated segments.
* indicates significance at $p \leq 0.05$



3.3.2.3 Individual Tissues

To refine the localisation of the JH hydrolysing enzymes modulated by methoprene, tissues from adults topically treated with methoprene were analysed.

Any effects on JHE and JHEH activities in particular tissues that could be attributed to solvents were assessed by comparing activities in tissues from untreated and solvent treated adults (data not shown). Ethanol significantly increased JHE activity in two tissues in female adults; the reproductive system and the head carcass (1.4 and 2.1 fold increases, respectively). Ethanol also increased JHE activity in male reproductive systems consistently in each assay but not significantly according to the statistical tests used. Because of the effects of solvents on JHE activities in some tissues, enzyme activities in tissues from methoprene treated adults were compared to those from ethanol treated adults.

JHEH activity in all tissues examined, except the digestive system, was too low and/or variable to determine whether methoprene treatment caused a significant effect on activity. In both males and females, the JHEH activity in the digestive system was not significantly affected by treatment with solvent or methoprene (data not shown).

Topical application of methoprene resulted in statistically significant increases of JHE activity, at the 95% confidence level, in two of the fourteen tissues tested, both of which were in males. JHE activity in the male reproductive system increased 1.3-fold and activity in male mouthparts increased 1.9-fold in response to methoprene treatment (Table 3.5, Figure 3.5). The effects identified in male reproductive systems and male mouthparts contributed to the effects conferred by methoprene in the male head and abdomen.

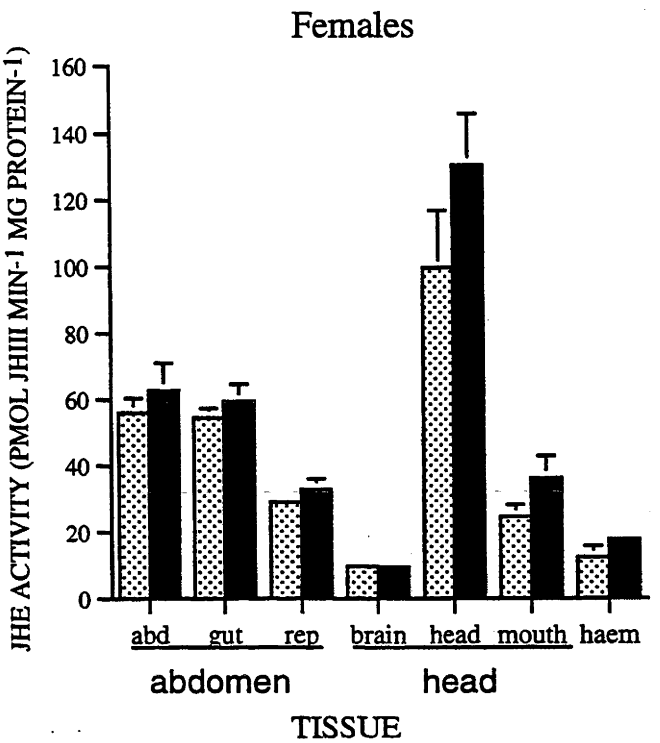
The effects of methoprene on JHE activity that were statistically significant do not explain the stimulatory effects of methoprene observed within segments. Inspection of the changes in enzyme activities in response to treatment suggests that methoprene did affect JHE activity

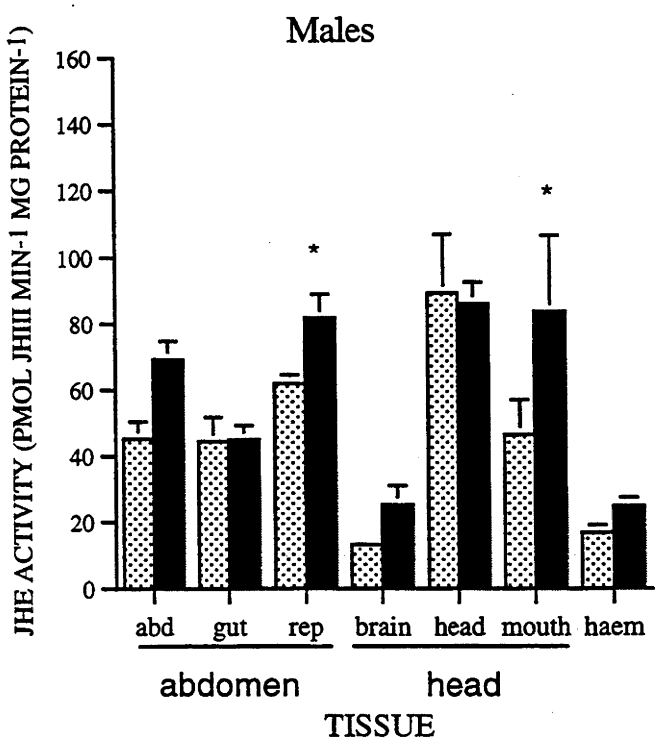
Table 3.5 Wilcoxon Signed Ranks were used to determine if there was significant increases in JHE activity in response to topical application of 5µg methoprene. Analysis was calculated by first subtracting control activity from treated activity then comparing data to one. 'Yes' indicates the increase in activity was significant at $p \leq 0.05$. Numbers in brackets denote mean fold increases in JHE activity.

	female	male
abdomen carcass	no	no* (1.7)
gut	no	no
reprod. system	no** (1.1)	yes (1.1)
head carcass	no** (1.4)	no
brain	no	no** (2.2)
mouthparts	no** (1.5)	yes (1.9)
haemolymph	no** (1.8)	no* (1.7)

* = one outlier from five replicates indicates no effect rather than stimulation.
** = one outlier from five replicates indicates repression rather than stimulation.

Figure 3.5 JHE activity per mg of protein in abdominal carcass (abd), digestive system (gut), reproductive system (rep), brain, head carcass (head), mouthparts (mouth) and haemolymph (haem), from one day old males and females treated with 5mg methoprene. Tissues from 30 adults represent one replicate and the means of 5 replicates are plotted. In some cases the standard errors do not exceed the thickness of the line. Stippled bars represent ethanol-treated tissues and solid bars represent JH tissues treated with methoprene.
* indicates significance at $p \leq 0.05$





in tissues other than the reproductive system and mouthparts of males. These effects are not statistically significant because of the variation in the data. There are two types of variation. Firstly, in the male abdominal carcass and haemolymph, data from one out of five replicates indicates methoprene has no effect on JHE activity rather than a stimulatory effect (i.e. the treated activity divided by the control activity equals one). Secondly, in female head carcasses, mouthparts, haemolymph and reproductive systems and male brain, data from one sample suggests that methoprene has a repressive, rather than stimulatory effect. Furthermore, in nearly all data sets derived from tissue dissections, large standard errors were present due to the variability of replicates. For example, the effect of methoprene on JHE activity in male brains ranged from a 0.8-fold decrease in activity to a 5.1-fold increase in activity. However, the mean effect on activity was a 2.2-fold increase.

After considering the data sets from all tissue dissections, it appears that methoprene stimulates the greatest increases in JHE activity in the following tissues: haemolymph of males and females (1.7- and 1.8-fold, respectively), the male abdominal carcass, brain and mouthparts (1.7-, 2.2- and 1.9-fold respectively).

3.4 Discussion

3.4.1 Regulation of JHE and JHEH Activities: Temporal Effects

3.4.1.1 Juvenile stages

This study suggests that JH does not regulate JHE and JHEH activities in juvenile stages of *D. melanogaster*. Despite testing the effect of JH on juvenile stages at up to eight time periods after each of these treatments only two minor effects were observed on JHE activity and no effects were found on JHEH activity. The significance of the relatively small effects on prepupal activity is unclear as one effect was inductive and the other repressive. Also, a

large dose of methoprene and a lengthy delay was required to detect the inductive response and the repression of activity was very small suggesting both responses may have been artefactual.

These results in the study of *D.melanogaster*, are consistent with those from the study of the mosquito, *C. quinquefasciatus*, in which methoprene treatment also did not affect JHE activity in larval or pupal stages. However, in prewandering larvae the peak of JH hydrolytic activity, which is due predominantly to JHEH activity, was eliminated in response to methoprene treatment. The pupal peak of JHEH activity was not affected (Lassiter *et al.* 1995).

These findings in the Diptera contrast with those reported for Lepidoptera in which JHE activity in larvae is generally induced in response to treatment with JH or JH mimics, and JHEH activity is unaffected (Jesudason *et al.* 1992; Kallapur *et al.* 1996). In some Lepidoptera, the prewandering peak of JHE activity is regulated directly by a "head" factor and indirectly by JH. JH treatment of prewandering larvae results in an increase in JHE activity of approximately 1.2-fold in *T. ni* haemolymph and up to 18-fold in *M. sexta* whole larvae (Sparks and Hammock, 1979; Jones *et al.* 1981; Venkatesh and Roe, 1988). However, these increased enzyme activities do not occur in starved larvae or larvae with a non-functional corpora allata (Sparks and Hammock, 1979; Cymborowski *et al.* 1982; Sparks *et al.*, 1983; Venkatesh and Roe, 1988). The prepupal peak of JHE activity in *T. ni* and *M. sexta* is directly induced by JH. In larvae with a functional corpora allata the increase in activity ranged from 1.3- to 3.4-fold while in larvae with a non-functional corpora allata, JH caused an increase in JHE activity of 16-fold in *T. ni* and 12.5-fold in *M. sexta* (Sparks and Hammock, 1979; Jones and Hammock, 1983; Sparks *et al.* 1983; Jesudason *et al.* 1992).

This comparison reveals that JHE activity is regulated by different factors in juvenile stages of dipteran and lepidopteran species even though JH and its hydrolytic enzymes have a similar developmental profile in the two orders. This may be related to the different roles

that JHE plays in these orders as a result of differences in the timing of commitment to metamorphosis (Riddiford and Ashburner, 1991; Riddiford, 1994).

JHEH activity does not appear to be regulated by JH in last instar larvae of *D. melanogaster*. In Diptera, the only effect of JH on JHEH activity reported has been in *C. quinquefasciatus*, at one particular life stage and this was a repressive effect (Lassiter *et al.* 1995;1996). In lepidopterans, no such effects have been reported.

3.4.1.2 Adult stages

This study suggests that JHE activity, but not JHEH activity, is regulated by JH in adult *D. melanogaster*. JHE activities in whole males and females treated with JH or a JH mimic were increased by up to 2.4-fold. Similar increases in JHE activity in response to methoprene treatment were recorded in *C. quinquefasciatus* adults and in JH treated prepupal Lepidoptera (Jesudason *et al.* 1992; Jones and Hammock, 1983; Lassiter *et al.* 1996). Although there is little information available on the effect of JH treatment on JHE and JHEH activities in adult Lepidoptera, one study suggested that JHE activity is not affected by treatment with JH (Venkatesh *et al.* 1988). Adult JHEH activities have not been observed to be affected in any insect species by JH or JH mimics.

The effects of methoprene and JHIII treatment on JHE activity in both whole and individual body segments of male and female *D. melanogaster* were similar, although the effects of methoprene were smaller and more variable. The physiological basis and functional significance of this difference in effect is unclear. However, studies into the *Met* mutant of *D. melanogaster* suggest that the effects of methoprene and JH are mediated via different binding proteins (Shemshedini and Wilson, 1990).

Although the effects of JH and methoprene on JHE activity in adults were significant they were relatively small, generally not leading to greater than two-fold increases in activity.

However, these studies were conducted using organisms with functional corpora allata and it is possible that there is a limit on production of JHE activity under these conditions and that increasing JH levels cannot further increase JHE activity. Allatectomising adults and thereby removing endogenous levels of JH prior to treatment may produce more pronounced effects. Evidence to support this proposal comes from the studies on lepidopteran larvae, described above. In lepidopteran larvae with a functional corpora allata, JHE activity is increased by topical application of JH by a similar amount to that observed in this study on *D. melanogaster*. In contrast, in larvae without a corpora allata, JHE activity is much more sensitive to topical applications of JH (Jones and Hammock, 1983; Jesudason *et al.* 1992).

This study on *D. melanogaster*, suggests that adult JHE activity is regulated by JH in a life-stage specific manner and it also demonstrates that JHE and JHEH activities are regulated independently. The significance of these findings was further explored by identifying tissues contributing to the overall adult effects.

3.4.2 Regulation of JHE Activity; Spatial Effects

Methoprene treatment of adults resulted in tissue- and gender-specific effects on JHE activity but not JHEH activity. These effects were broadly distributed throughout the organism, in all female and male segments, with the exception of the female abdomen. Surprisingly, methoprene and JH treatment had their most profound effects on JHE activity in the heads and thoraces of males and females; segments where such effects were relatively unexpected.

The size of the effects on JHE activity caused by methoprene and JH treatment in segments and tissues were consistent with those seen in whole adults. Most effects were less than two-fold increases in activity. The greatest increases in JHE activity were detected in tissues from the heads of females and males. JH has been implicated in few processes in the head of adult insects so it is difficult to speculate on the significance of these tissue and sex specific effects, although one possibility is pheromone reception (Wyatt and Davey, 1996). JHE

activity in haemolymph of both males and females was also increased by a relatively large amount following methoprene treatment. This is another unexpected result as the haemolymph of dipterans is generally considered to have low levels of JH hydrolytic activity (Weirich and Wren, 1976; Klages and Emmerich, 1979; Wilson and Gilbert, 1978; Shemshedini and Wilson, 1988). Finding a relatively large increase in JHE activity in such tissues demonstrates that there may exist the potential for higher levels of JHE activity in tissues which may be biologically significant at some stage of development as yet unidentified.

The reproductive system extracted from the female abdomen showed an insignificantly small increase in JHE activity following methoprene treatment. This was an unexpected result as changes in JH titre are considered to have several roles in female reproduction in the Diptera (Riddiford, 1994; Wyatt and Davey, 1996). Khlebodarova *et al.* (1996) have proposed that in *Drosophila*, JH is regulated locally in specific areas of the reproductive tissues by rapid synthesis and degradation. Under this hypothesis it is predicted that JHE, the major hydrolytic enzyme in ovaries at least, would be responsive to JH. This study on *D. melanogaster* suggests that methoprene treatment does not greatly affect JHE activity in reproductive systems of virgin females.

A possible explanation for this surprising outcome is that the females in this study were young and unmated. Development of eggs in unmated females differs from those of mated females, particularly with regard to the amount of yolk protein being produced. JH is known to stimulate production of yolk proteins but it is also believed that JH must be low so that termination of egg maturation and oviposition occurs (Jowett and Postlethwait, 1980; Shapiro *et al.* 1986; Wyatt and Davey, 1996). It is possible that JHE activity responds differently to JH in reproductive tissues of mated and unmated females.

The abdominal carcass and reproductive systems of males showed increased JHE activity following methoprene treatment. It is unclear why the abdominal carcass would have such

high stimulation of JHE activity, however, increases in JHE activity in the reproductive system are consistent with reported effects of JH on these tissues. Physiological effects of JH on the male reproductive system include stimulation of protein synthesis in the male accessory glands of *D. melanogaster* and *A. aegypti* (Yamamoto *et al.* 1988; Fernandez and Klowden, 1995). JH has also been found to stimulate the activity of one particular protein, Esterase 6, approximately two-fold, in the ejaculatory duct of *D. melanogaster* males (Healy, personal communication). These data are consistent with JH having a role in male reproductive tracts (Wyatt and Davey, 1996).

This study demonstrates a clear role for JH in regulating JH hydrolytic activity. JH has differential effects on the JH hydrolysing enzymes, JHE and JHEH, and it regulates JHE activity temporally, spatially and sex specifically. Several mechanisms may lead to differential regulation of JHE and JHEH activities. There is evidence that isoforms of JHE exist in *D. melanogaster* allowing the possibility that tissue specific isoforms are regulated differentially by JH (Campbell *et al.* 1992). Other possibilities are that JH is confined to particular subcellular fractions and so can affect JHE activity only in those fractions. Specific factors, such as binding proteins, receptors and DNA sequences, necessary for expression of JHE activity may also provide developmental, tissue and sex specificity for regulation by JH.

One final point of consideration in this study, is that JH treatment of adult females and males is known to increase protein production in reproductive tissues (Yamamoto *et al.*, 1988; Wyatt and Davey, 1996). Since JHE and JHEH specific activities were used in comparisons of treated and untreated organisms it would be reasonable to assume that parallel increases in protein content and JH hydrolytic activity would obscure any JH or methoprene effects. However, comparison of protein concentrations in whole adults, segments or tissues did not indicate increases in protein concentration before and after treatment (data not shown).

Chapter Four

General Discussion

The major aims of this study on *D. melanogaster* were 1) to consider the roles that JHE and JHEH activities play in juvenile and adult life stages by determining the expression patterns of these enzymes and 2) to investigate the regulation of JHE and JHEH activities. One level of regulation in particular was examined; the regulation of JHE and JHEH activities by JH in *D. melanogaster.* In Lepidoptera, JH regulates JHE activity and visa versa.

Summary of Results

At ecdysis to the third-instar, JH hydrolysis was low. In prewandering larvae, peaks of predominantly JHEH, but also JHE activities were observed. Both enzymes had low activities by the wandering stage. About one hour prior to pupation a second peak in JHE activity was observed. In adult females and males, JHE specific activity was high at eclosion then fell to a constant lower level at one day old. The JHEH specific activity in adults was relatively constant from eclosion to two days old.

The spatial profiles of JHE and JHEH activities in females and males showed that enzyme activities were highest in abdominal segments and JHE activity was present in all tissues examined. In females, JHE activity was highest in the reproductive and digestive tissues and the abdominal carcass, which contains most of the fat body. In the reproductive tissues, JHE activity was highest in the ovaries. In males, JHE activity was also detected in all tissues and was highest in the reproductive and digestive tissues. In reproductive tissues, activity was highest in the testes. JHEH activity in females and males was highest in the digestive tissues.

Methoprene treatment in larval or pupal life stages did not affect JHE and JHEH activities. However, one-day-old adults treated at eclosion with JH or JH mimics had increased JHE

activity, but not JHEH activity. In females, the increase in JHE activity was due to increases in the head and thorax, while in males, increases were observed in all segments. Tissues with the highest increases in JHE activity after treatment, were the haemolymph of males and females and the male abdominal carcass, brain and mouthparts.

4.1 The Roles of JH Hydrolytic Enzymes in *D. melanogaster*

In most insects species, the role of JH hydrolytic enzymes in juvenile stages is to regulate the titre of JH by removing all traces of JH from circulation. Comparisons of developmental profiles of JH titre and synthesis with those of JHE and JHEH activities provide evidence that JH hydrolytic enzymes also play this role in juvenile life stages in *D. melanogaster*. These profiles are similar to those found in other insects, particularly Lepidoptera (Campbell *et al.* 1992; Roe and Venkatesh, 1990). The profile of JH hydrolysis in the final instar through to pupation was similar to previous reports in many insects and inversely correlates with the JHIII titre and JH biosynthetic activity of the ring gland (Bownes and Rembold, 1987; Sliter *et al.* 1987; Richard *et al.* 1989a).

In adult *D. melanogaster*, the JHE and JHEH activities presumably have the same general role in regulating JH titre, as in juvenile stages. However, the evidence for this role in adults is based mainly on the comparison of tissue distributions and abundance of JHE and JHEH activities with tissues in which JH has a physiological effect, rather than comparisons of developmental profiles. The developmental profiles of JHE and JHEH activities in adults suggest there is no relationship between the JH hydrolytic enzymes and whole body JH titre and biosynthesis. The tissue distribution of JH hydrolytic enzymes in adults, suggests instead a JH/JH hydrolysis relationship occurs in specific tissues, particularly reproductive tissues.

In *D. melanogaster* females, it has been suggested that JH hydrolysis is important in reproduction for regulating the titre of JH which needs to change rapidly so that continuous

egg production and maturation can occur (Khlebodarova *et al.* 1996). The results in this thesis suggest that JH hydrolysis indeed has a role in reproduction in *D. melanogaster.* JH hydrolytic enzymes were detected in all tissues examined and were highest mainly in tissues where JH is believed to play its major roles, in particular reproductive tissues. JHE activity was at high levels, not only in male and female reproductive tissues but in females, high levels of activity may also be present in the fat body. The fat body is a tissue intimately involved in female reproduction and requires JH for vitellogenesis and subsequent egg development.

In other species, JH hydrolysis has also been found to play a role in reproduction. In female *A. aegypti* and *C. quinquefasciatus*, JH hydrolysis is believed to be important in reducing the JH titre for reproductive development, in particular vitellogenesis (Shapiro *et al.* 1986; Lassiter *et al.* 1994; 1996). In virgin females of *T. ni*, JHE activity remains high after eclosion but declines after mating. JHE activity is believed to keep JH at low levels in virgin females so that egg development is suppressed; a high JH titre allows vitellogenesis to occur (Venkatesh *et al.* 1988).

4.2 Levels of Regulation of JHE and JHEH Activities

JHE and JHEH activities appear to be regulated at a number of levels; developmentally, spatially, sex-specifically and also differentially. Such findings have also been observed in other insects, including other Diptera and Lepidoptera (Lassiter *et al.* 1994; 1995;1996; Shapiro *et al.* 1986; Roe and Venkatesh 1990; de Kort and Granger, 1996). Many levels of regulation for JHE and JHEH activities may be necessary for regulation of JH titre, since the regulation of JH titre is essential to the successful development of most life stages in insects.

The differential regulation of JHE and JHEH activities may provide an additional level of complexity to the regulation of these enzymes. In juvenile stages, the activities of JHEH and JHE fluctuate substantially. In prewandering larvae, JHEH activity is predominant while

JHE activity is predominant in prepupae. The significance of this is unclear but it is possible that differential regulation of JHE and JHEH activities is an important feature of the specificity and the subcellular distribution of these enzymes and the ultimate fate of JH after hydrolysis.

The specificity of JHE and JHEH for JH analogues may be one reason for the differential regulation of these enzymes. The biosynthesis of three JH analogues from the ring gland and corpora allata of *D. melanogaster* have been reported; JHIII bisepoxide, JHIII and methyl farnesoate (Richard *et al.*, 1989a; Alteratz *et al.*, 1991). In *P. regina*, specific ratios of JH analogues were found to have a greater biological effect than individual JH analogues suggesting that blends of JH analogues may be biologically relevant (Yin *et al.* 1995). It is possible that the titres of these JH analogues are regulated not only by biosynthesis but by the specificity of JHE and JHEH activities to hydrolyse them. JHE and JHEH hydrolysis of JH analogues may be regulated by interaction with JHBPs. Interestingly, it was found that lipophorin from *L. cuprina* bound JHIII with high affinity and binding was not displaced by JHIII bisepoxide (Trowell *et al.* 1994). In *D. melanogaster*, lipophorin is believed to inhibit JH hydrolysis by binding JH, making it unavailable for hydrolysis (Campbell *et al.* 1998). Such evidence suggests that lipophorin may regulate the availability of JH analogues for hydrolysis. Coupled with the possibility that JHE and JHEH activities specifically hydrolyse JH analogues, regulation of JH titre by hydrolysis may be a reason for the differential regulation of these enzymes. A similar relationship is believed to exist in moths but the interaction of JH, JHBP and JHE may have more levels of complexity (Touhara *et al.* 1995).

Subcellular distribution of JH, JHE and JHEH may also explain the independent regulation of the enzymes. In prewandering larvae of *D. melanogaster*, the peak of JHEH activity is mostly membrane-bound; in the microsomal and mitochondrial fractions, while most of the pupal JHE activity is soluble (Casas *et al.* 1991, Harshman *et al.* 1991; Campbell *et al.* 1992). The subcellular distribution of the enzymes may reflect the subcellular distribution of JH at these life stages and suggests that specific factors with unique distributions are

involved in the differential regulation of JHE and JHEH activities.

The fate of JH after hydrolysis by JHE and JHEH is different and may reflect the biological importance of hydrolysis of JH by a particular enzyme. Although the recycling of JH acid in Diptera is unreported, JH acid can be recycled back to JH by JH methyltransferase in tissues, such as imaginal discs in moths (Sparagana *et al.* 1985). As such, JHE hydrolysis of JH to produce JH acid is reversible. In addition, recent reports indicate that in *M. sexta* larvae, JH acid has a hormonal function; to induce vitellogenin, a function which cannot be undertaken by JH, except in cooperation with other hormones (Ismail *et al.* 1998). In comparison, the fate of JH diol following JHEH hydrolysis of JH is irreversible and JH diol has no known hormonal function.

4.3 Regulation of JHE Activity by JH

The different levels of regulation imposed on JHE and JHEH activities suggest that there are specific and unique factors that regulate each of these enzymes. This study provides evidence that JH regulates JHE, but not JHEH activity, developmentally, spatially and sex-specifically. While JHE is also regulated by JH in a similar manner in moths, one major difference between flies and moths is that regulation of JHE activity by JH occurs at different life stages.

4.3.1 Temporal Regulation of JHE activity by JH

In larval and pupal life stages in lepidopterans, the circulating titre of JH is primarily controlled by a decrease in biosynthesis but JHE activity is believed to play a crucial role in "scavenging" the final traces of JH (Jones, 1995). In last-instar larvae, JHE activity is regulated temporally and spatially by JH, neuroendocrine and environmental factors (Roe and Venkatesh, 1990). A regulatory feedback mechanism between JH and JHE has been documented whereby JHE activity regulates JH titre and visa versa (Jones and Hammock,

1983). The reason for JH regulating its own degradative activity in lepidopteran larvae, is possibly because it is the fastest and most efficient method of regulating it own titre (Jones and Hammock, 1983). However, JH does not induce JHE activity in adult stages (Roe and Venkatesh, 1990).

One of the most intriguing results from this study on *D. melanogaster* is that the JH mimic, methoprene, influenced JHE activity in life stages not anticipated. Although it appears that JHE plays a similar "scavenging" role in the juvenile stages of dipterans, as in lepidopterans, results from this study on *D. melanogaster* suggest that JH does not regulate hydrolytic activity in the juvenile stages. Instead methoprene influenced JHE activity in adult stages only. A similar study on another dipteran, *C. quinquefasciatus,* also found that methoprene influenced JHE activity in adults and not larval stages, but JHE activity was decreased not increased (Lassiter *et al.* 1996). It is not apparent whether the predominance of JHEH over JHE activity in both juvenile and adult stages of *C. quinquefasciatus,* explains this difference in regulation (Lassiter *et al.* 1994; 1995).

There are a number of physiological reasons why JH may regulate JHE activity at different life stages in the Lepidoptera and Diptera and these differences in physiology may reflect the significance of the required level of control of JH titre. In juvenile stages, the most obvious physiological difference associated with JH between the two orders is in the timing of commitment to metamorphosis. JH treatment causes subtly different physiological effects in juvenile life stages of moths and flies even though JH and JH hydrolysis have a similar developmental profile in the two orders. In Lepidoptera, if the JH titre is maintained in the prewandering larvae, commitment to metamorphosis does not occur (Cymborowski *et al.* 1982; Jones and Click, 1987). However, higher dipteran species become committed to metamorphose long before the wandering stage of development. Hence, JH treatment in the last larval instar does not affect commitment to metamorphosis (Riddiford and Ashburner, 1991; Riddiford, 1994). This suggests that in the Diptera, the decline in JH titre at this life stage has a different developmental significance compared to the Lepidoptera (Riddiford and

Ashburner, 1991; Campbell *et al.* 1992).

The prepupal peak of JH hydrolysis in lepidopteran species is also critical for successful metamorphosis. Treatment with JHE inhibitors or JH at this stage disrupts metamorphosis and normal development of adult structures (Jones and Hammock, 1983). In Diptera, JH treatment of prepupae does not halt pupation but it does cause abdominal histoblast cells to produce abnormal cuticle in adults suggesting that it is important that there is no JH present at this stage (Postlethwait, 1974; Riddiford and Ashburner, 1991). Histoblast cells become sensitive to JH after pupariation so presumably JHE and JHEH activities play some role in determining the outcome of pupation or subsequent adult development since the peak of JHE activity, at least, is just prior to pupation. It therefore appears that JHE activity is regulated by different factors in moths and flies at particular life stages and this may relate to the different developmental programs in Lepidoptera and Diptera.

In adult life stages of dipterans and lepidopterans, differences in physiology may also explain differences in regulation of JHE activity. This is illustrated by the markedly different effects of JH on reproductive development in females, in the two orders. Vitellogenesis in both orders begins before eclosion but in the majority of Lepidoptera, JH does not affect vitellogenesis at this life stage. However, in dipterans, vitellogenesis is dependent upon JH (Wyatt and Davey, 1996; Bownes and Reid, 1990). Furthermore, in nearly all insects, vitellogenesis occurs exclusively in the fat body while in higher Diptera, including *D. melanogaster*, vitellogenesis occurs in both the fat body and the ovaries (Wyatt and Davey, 1996). In *D. melanogaster,* vitellogenesis in ovaries is regulated by JH alone while vitellogenesis in fat body is regulated by JH and ecdysone (Wyatt and Davey, 1996). Although the role of JH catabolism, if any, is unknown in vitellogenesis, such differences may explain variations in JHE regulation between these orders.

4.3.2 Spatial Regulation of JHE activity by JH

Following the characterisation of the spatial distribution of JHE activity in adults, it was a relatively unexpected result that JH or methoprene treatment caused increases in JHE activity in segments and tissues characterised as having low JHE activity. The increased JHE activity measured in methoprene-treated females was isolated to the thorax and head; segments which contained only a small proportion of the total JHE activity detected in whole individuals.

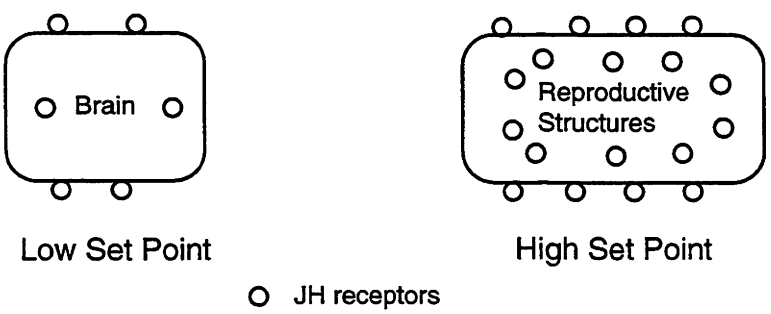
This anomaly was also evident in males, although to a lesser extent than in females. Males treated with JH or methoprene had essentially equal increases in JHE activity in all segments even though it was the abdominal segment where JHE activity was highest in untreated males. In contrast to females, there was a significant increase in JHE activity in male reproductive tissues but this increase was small in comparison to the increases observed in other tissues, such as male mouthparts.

These unexpected results in females and males suggest that the regulation of JHE activity is complex. One possibility is that there is some limit to the amount of JHE activity that can be produced in a tissue and that JHE activity is relatively tightly controlled. Tissues with the greatest increases in JHE activity due to methoprene treatment i.e. haemolymph, the male abdominal carcass, brain and mouthparts, were those not obviously associated with processes in which JH is known to be involved. May be because of this, the activities of JH hydrolytic enzymes in these tissues may not be as tightly controlled, allowing a greater flexibility in the levels of JHE activity. A limit to the amount of induced JHE activity in a tissue may also explain the relatively small increases in activity, observed after treatment with JH or methoprene. It is possible that larger increases in JHE activity would be observed in adults lacking a functional corpora allata, in adults having reduced JH, such as *apterous* mutants, or in adults treated with precocene, an inhibitor of JH synthesis (Staal, 1986; Shtorch *et al.* 1995).

An alternative explanation is that there is differential regulation of JHE activity among tissues. JHBPs have been detected in reproductive tissues (including in females, the ovaries and fat body and in males, the accessory glands; Roe and Venkatesh, 1990) and presumably, it is in such tissues that JH is targeted and becomes concentrated. These tissues are also where JH is known to have roles in adults. In *D. melanogaster*, reproduction is continuous and JH is likely to be in constant demand. This suggests a constant level of JHE activity is necessary, rather than the rapid increases and decreases that have been observed in larval and pupal life stages. Therefore, inductive regulation of JHE activity by JH in these tissues may be unnecessary and may even be detrimental to reproduction.

Alternatively there may exist set points that must be reached before JHE activity becomes regulated by JH i.e. the density of JH receptors in reproductive tissues may severely limit the concentration of free JH and induction of JHE activity may occur only when the titre of free JH reaches a certain set point (Figure 4.1). Such a set point may not have been reached in reproductive tissues with the concentrations of methoprene used in the experiments described in chapter three. However, in other tissues such as the brain, mouthparts and haemolymph, where the greatest increases in JHE activity were observed, a different level of regulation of JHE activity may be necessary. The density of JH receptors is likely much lower in such tissues so the concentration of free JH would be greater. High concentrations of free JH may be destructive in these tissues and so tight regulation of JHE activity may be necessary. Subsequently, the set point of free JH may have been reached in the experiments undertaken, allowing induction of JHE activity by JH to occur. Additional evidence of the existence of either of these situations; that JHE activity is not regulated by JH in some tissues or that set points of free JH exist, is that a dose response was not observed in adults treated with three different concentrations of JH (see section 3.3.2.1). This result is evidence that the effect of JH on JHE activity is either all or none.

Figure 4.1. Representation of possible JH receptor density in brains and reproductive structures of *D. melanogaster*. The greater the number of receptors, the lower the titre of free JH within the tissue at any one concentration. The set point indicates that when free JH titre reaches a certain level, JHE is induced in that tissue. This model would permit differential regulation of JHE by various tissues.



The regulation of JHE activity in digestive tissues may be different again. These tissues may contain constitutive high levels of JH hydrolytic enzymes ready to degrade any JH taken into the body externally or filtered from the rest of the body.

JH may also regulate JHE activity in cooperation with other factors, tissue specifically. For example, in lepidopteran larvae, brain factors have been identified that act coordinately with JH to regulate JHE activity. These brain factors modulate the response of JHE to JH by having either a stimulatory or inhibitory influence on regulation, depending on the species (Sparks and Hammock, 1979; Sparks *et al.* 1983; Venkatesh and Roe, 1988). Such factors may have tissue specific effects in adult *D. melanogaster*. If such factors originate from the head, isolating tissues from the brain, either by neck ligation or organ culture would limit their presence. In organ culture it would also be possible to measure the effect of addition of brain extracts into the medium.

4.4 Regulation of JHEH activity by JH

In the insects studied to date, including this study on *D. melanogaster*, none have shown an increase in JHEH activity in response to treatment by JH or a JH mimic, suggesting that JHEH activity is not regulated by JH. One of the few reports of an effect of JH on JHEH activity was in the last larval instar of *C. quinquefasciatus,* in which methoprene eliminated the normal peaks of JHEH activity. No effect of methoprene on JHEH activity was detected in pupae however (Lassiter *et al.* 1995). Possibly another signal such as a brain factor, similar to the brain factor that regulates JHE activity in prewandering lepidopterans, also regulates JHEH activity in insects.

4.5 Future Directions for Examining the Regulation of JHE and JHEH Activities

In the Lepidoptera, molecular biology has provided insight into factors that regulate JHE activity. To date, the isolation of the JHE gene in *D. melanogaster* has been elusive. Such a break-through would allow investigation of JHE regulation at the molecular level similar to what has been accomplished in *T. ni* (Jones *et al.* 1996; Jones *et al.* 1998). However, other experiments would possibly also give insight into the regulation of JHE activity by JH. Organ culture of reproductive tissues in particular, would provide insight into the levels at which JH regulates JHE. Similar work has been reported in the accessory glands of *D. melanogaster,* in which JH-induced production of accessory gland proteins was found to occur via a second messenger system (Yamamoto *et al.* 1988)

Investigating factors other than JH, involved in the regulation of JHE activity in particular, could also be based on research already completed in various species of Lepidoptera, such as ligation experiments which identified a brain factor involved in the regulation of JHE activity (Venkatesh and Roe, 1988).

4.6 Conclusions

The developmental and spatial profiles of JHE and JHEH activities in *D. melanogaster*, suggest that the role of these enzymes in last instar larvae, pupae and adult stages is to regulate the titre of JH, the same role these enzymes have in Lepidoptera. In adult life stages, the spatial expression patterns of JH hydrolytic enzymes suggest that JHE in particular , is involved in regulation of JH in the reproductive tissues of both females and males, which is similar to other insects.

JHE and JHEH activities appear to be regulated at a number of levels; temporally, spatially, sex-specifically and differentially which is also what is observed in other dipterans and lepidopterans. The specific factors regulating these enzymes are largely unknown, however,

in this study in *D. melanogaster*, JHIII and a JH mimic, methoprene were observed to increase the activity of JHE suggesting that *in vivo* JH regulates JHE activity. A similar feedback mechanism has been documented in lepidopterans but it is known to exist only in juvenile, not adult life stages. In *D. melanogaster*, such a feedback mechanism appears to exist in adult life stages only.

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